

## Don't leave it to the market-place

**The collapse of the British government's attempts to privatize its observatories should not be the end of the story. But any future government must take heed of the lessons of a severe, and costly, embarrassment.**

Two key issues confront any government concerned to make the best use of scarce resources for research. The first is prioritization. The second, once priority areas have been chosen, is to find ways of spending the money in the most cost-effective way.

Shortly after the Conservative party won the last British general election, it decided on radical new approaches to both tasks. For the first, the government set out to build a consensus on priorities between the producers and users of research through the process that was given the ungainly title of Technology Foresight. The success of this strategy has been markedly greater than that of the government's efforts to tackle the second. These have been based on an overzealous approach — firmly located in a belief in the overriding powers of the market-place — that tries to draw a firm dividing line between those bodies whose job is to decide what research public money should be used to 'purchase' and those that seek to provide the 'goods' that meet this description.

There are, of course, areas in which this approach can reap substantial dividends, and has already done so. Most of these are cases in which research priorities can be defined by relatively clear, and often short-term, objectives. When it comes to government institutions that support more basic science, however, the situation is different. Goals and targets cannot be so precisely defined. And there is a fundamental discrepancy between the year-to-year timescale on which profit-oriented bodies tend to operate, the five-yearly timescale of most politicians and the much longer timescale that substantial scientific achievement requires.

Such a discrepancy in timing has, as much as anything, been the rock on which efforts to transfer the management of Britain's national observatories into private hands has foundered (see page 391). Few have needed persuading that there is much to be gained in principle by such a move. The main stumbling-block appears to have been the need to provide for the pension rights of employees. Here, the Treasury appears to have seen no short-term benefit in digging into its pockets for the £12 million or so which, in the long-term, it will still be obliged to provide. Correctly, its demand that this money be taken out of the annual science budget has been firmly resisted by the Office of Science and Technology.

The resulting stalemate would be farcical if up to £2 million had not already been committed in legal fees on the proposed transfer (some of it admittedly to cover redundancies and separate legal difficulties). This is money that would otherwise have been spent on research at a time when the Particle Physics and Astronomy Research Council (PPARC) is already almost bankrupt. PPARC's experience has certainly given the government a brutal taste of reality, as well as justified embarrassment, as it contemplates the similar transfer to private management of a raft of research council institutes. The total pension commitment of these runs into hundreds of millions of pounds. Taking that type of money out of the science budget would have a devastating effect.

As a thoughtful report published this week by the House of Commons Select Committee on Science and Technology points out, there is much to applaud in the government's desire to ensure that public money is spent on science in the most cost-effective way. The big question mark is whether the market-place alone is the best way of achieving this. The committee's answer, appropriately enough, is that there are cases in which this is true. But in

others the answer is clearly no. In such circumstances, a common interest in good science means that strategic alliances between those who decide scientific priorities and those who pursue them must be maintained.

That, of course, has long been the argument of the opposition Labour party. If, as widely expected, it is returned to power in the general election due next May at latest, it will have the opportunity to put its money where its mouth is. But it will have to avoid the opposite traps offered by the rigidities and vested interests of the status quo. Various imaginative alternatives exist, such as making more use of university-based groups as the managerial bridge between the taxpayer as the 'customer' and the scientist as the 'provider' of basic research. But an uncritical commitment to continued state control would be as unproductive as an equally uncritical embrace of market solutions has already turned out to be. □

## News story that wasn't

**A biotechnology company is developing the non-informative press release into a fine art.**

MANY readers of this issue of *Nature* will no doubt be pleased to be informed of two new genetic links to a form of diabetes (maturity-onset diabetes of the young, or MODY). MODY patients suffer from a relative inability of the body to produce insulin in response to glucose stimulation. The new work (pages 455–460) reveals links between particular gene defects and two types of MODY, but, more significantly still, that the defective genes code for particular proteins that regulate gene transcription. The specific identification of those mutant proteins opens up new lines of research (see pages 407–408).

Interested science journalists will have been aware of all of this a week ahead of the publication date, as *Nature* told them about it in its weekly (embargoed) press release. Some of those journalists may also have received a press release issued a week previously by Millennium Pharmaceuticals Inc. That company, based in Cambridge, Massachusetts, specializes in identifying genes responsible for major diseases. Their work includes a \$70-million collaboration with Hoffmann-La Roche in obesity and type-II diabetes.

And, apparently, that work has borne fresh fruit. Millennium says that it "has identified a gene implicated in the development of type II diabetes". The press release refers to "diverse population sources" of the DNA, and "positional cloning techniques". But that, as far as the science is concerned, is it. We are told that, commendably, details of the work are being withheld pending publication in a peer-reviewed journal. But also that Hoffmann-La Roche has already been sufficiently impressed by the work to hand over a "milestone payment" — amount unspecified. On the other hand, caution is necessary because: "This press release contains forward looking statements... subject to risks and uncertainties that may cause actual results to differ materially from those stated". That standard legal disclaimer appears unusually apt.

Readers can speculate for themselves on the purpose of such a release. Business news it may be, but hard science news it isn't. □



# 'Extra costs' force UK to terminate bid to privatize astronomy facilities

**London.** The UK Department of Trade and Industry is abandoning its current efforts to find private managers for British telescopes in the Canary Islands and Hawaii, as well as for the Royal Greenwich Observatory and the Royal Observatory of Edinburgh. The decision represents an embarrassing setback to efforts to 'privatize' as much government sponsored research as possible.

A statement issued late last Friday (30 November) by Ian Taylor, the minister responsible for science, said that a decision had been made to terminate the tendering process for management contracts for these facilities, which are operated by the Particle Physics and Astronomy Research Council (PPARC). This was because "difficulties which have significant financial and legal implications remain to be resolved".

Although officials refuse to give further details of these difficulties, it is generally accepted that they focus on the costs of 'pensions crystallization' — the fact that any private contractor taking on the contracts will expect a substantial lump sum payment from the government to cover pension commitments (see *Nature* 384, 201; 1996).

The decision to suspend the tendering process, for which several potential bidders were lined up, came three days after the announcement of next year's science budget (see below) in which the Treasury does not appear to have made any extra money available to cover the costs to the research councils of preparing their institutes for possible transfer to private management.

It also coincides with a critical report from the House of Commons Select Com-

mittee on Science and Technology, which has been holding hearings into the so-called 'Prior Options' exercise. This is the most recent of three government reviews aimed at identifying laboratories and research council institutes for privatization.

In its report, published on Monday, the committee says it does not question the

Many astronomers would welcome a management structure independent of PPARC.

But there is widespread concern that the substantial legal costs already incurred, said to amount to about £2 million, have had to come out of the research council's operating funds. The pain to astronomers has been increased by the fact that PPARC is already heavily strapped for funds.

"We are extremely angry," says Phil Charles, head of the department of astrophysics at the University of Oxford, and a member of one of several panels set up to prepare for the tendering process. "We should have said that we would only take on the work of these panels once we had received a guarantee that these costs would be covered from elsewhere."

PPARC's embarrassment has been seized on by Adam Ingram, the opposition Labour Party's shadow minister for science and technology. Ingram said on Monday that the decision shows that the government was "clearly in disarray" over the prior options review policy.

Ingram claims that the government's apparent U-turn underlines his own party's position, namely that "there are no advantages to the science and research base of this country in the privatization of our world-renowned national institutions such as the Royal Observatories and other research institutions". Ingram says that if — as now seems highly likely — Labour is elected to power next year, it will make its own assessment of the need for management changes "based on science, not ideology".

The government itself remains committed, at least in principle, to continuing along the path it has laid out. Taylor's statement said that tendering for the management contracts "will proceed once the difficulties have been resolved and the costs and benefits reassessed".

But the government still faces the thorny question of how to meet the pension commitments of many other research institutes which, in principle, it would like to see privatized. There is a widespread feeling that, as with the observatories, crucial decisions on at least some of these may now be postponed until after the general election.

The select committee made clear its support for a more cautious approach. "At a time when research councils can fund far fewer than 50 per cent of the alpha-rated proposals they receive, costly reorganization should not be undertaken without a balance of evidence in its favour," says the committee's report.

**David Dickson**

IMAGE  
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**In the dark: the Infrared Telescope in Hawaii is one whose future management is unclear.**

wisdom of seeking to achieve the maximum return on public investment. But it adds: "We are not convinced that the current reviews have been driven by concern for science, rather than financial considerations."

The committee says that the scale and conduct of reviews of research council institutes seen as possible candidates for privatization has been "profoundly unsatisfactory", adding that any possible benefits for these institutes "have almost certainly been overshadowed by the disruption caused".

The government's case has not been helped by its experience of trying to find a private manager for its astronomy facilities.

## British science to stay in steady state

**London.** The British government announced last week that next year's science budget will be at the same level as this year, namely at £1.3 billion (US\$2.1 billion).

This figure is in line with plans for research published in July in the government's so-called *Forward Look*. But it means that, in real terms, funding for science will fall by about 2.5 per cent, the expected level of inflation. And the outlook for future years, according to these plans, looks even bleaker.

Nevertheless, given the general pressure on the government to cut public spending in what will be the last budget before the next general election, expected next spring, the general reac-

tion has been pragmatic. "This might have been worse," says John Mulvey, secretary of the pressure group Save British Science. He says that the budget settlement reflects vigorous efforts by the Department of Trade and Industry's Office of Science and Technology to ward off deeper cuts.

There is also some relief for university researchers in the government's decision to provide an extra £100 million for recurrent spending by universities. This is being widely seen as an implicit acknowledgement by the government that it made a serious mistake last year when it cut £450 million from the universities' capital equipment budget over three years.

**D. D.**



# Did UK scientist give France vital clues about H-bomb?

**Paris.** Historians reacted sceptically this week to controversial claims by a retired French weapons scientist that a British atomic scientist provided crucial information that helped France to explode its first hydrogen bomb in 1968.

Writing in *La Recherche*, the monthly science magazine, Pierre Billaud — who supervised the explosion of France's second H-bomb later the same year — asserts that the UK scientist approached André Thoulouze, the military attaché at the French embassy in London in 1967. The magazine identifies the scientist as Sir William Cook, who was the head of the British H-bomb project at Aldermaston in the 1950s.

Billaud says that the scientist approached Thoulouze with the question "Your H-bomb, it's not coming along is it?" and later advised the French: "Don't look for complications. Try a simple design". According to Billaud, the British would have known that the French were pursuing the wrong course from analyses of fallout from atmospheric tests.

The UK scientist later gave Thoulouze other information, writes Billaud. Although no papers changed hands, this was sufficient to indicate to the French that they should use X-rays from a fission device to compress the thermonuclear fuel and trigger fusion, instead of pursuing two more complicated options. At the same time, Billaud played down the importance of the British contribution, arguing that it allowed France to save two months at most, as trials of the X-ray techniques were already planned.

But Lorna Arnold, official historian of the UK Atomic Energy Authority, says she is "sceptical" that Cook would have passed on such information. She argues that it would have been "inconceivable" for him to have done so without authorization from the government. And Britain would have been extremely unlikely to have acted without the blessing of the United States, she argues.

"Something doesn't add up," says Arnold, pointing out that, under a 1958 agreement with the United States, Britain was forbidden to pass information on nuclear matters to third parties. "There is something mysterious about [the claims]." Arnold's thinking is shared by André Finkelstein, a scientist at the French Atomic Energy Commission (CEA) who worked on the CEA archives before his retirement. He dismisses the possibility that Britain helped France as being without foundation.

Both speculate that the claims may be related to French political infighting as to who should receive credit for developing the H-bomb. The most widely accepted version originates from statements by Alain Peyrefitte, General Charles de Gaulle's minister of research, and atomic and space affairs. Peyrefitte attributed the critical insight needed to develop the bomb to Roger Dautray, who was then a young physicist and is currently high commissioner of CEA.

Billaud's claims implicitly query Dautray's leading role, because they reaffirm comments made in 1984 by Jacques Chevallier — then head of CEA's military wing, DAM — that the major breakthrough was made by Michel Carayol, a young military engineer credited with recognizing the need for X-ray compression of the thermonuclear fuel.

Philippe Bergereau, a spokesman for CEA, says that the development of the French H bomb was "a team effort, and the paternity cannot be attributed to any individual".

**Declan Butler**

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Cook: identified as possible contact.

Universal Pictorial

## New US air pollution regulations are set for a bumpy ride

**Washington.** The scientific debate over the health effects of airborne particles and ground-level ozone is set to become more public and acrimonious following a proposal last week by the US Environmental Protection Agency (EPA) to tighten regulations on both pollutants.

The EPA's proposed rule would lower the standard for ozone from 0.12 parts per million (ppm) measured over a one-hour period to 0.08 ppm over eight hours. It would regulate fine (2.5 microns and smaller) particles in the air for the first time, restricting their concentrations to 15 micrograms per cubic metre annually and 50 micrograms per cubic metre daily.

Both recommendations are based on controversial scientific evidence. The EPA's Clean Air Scientific Advisory Committee (CASAC), for example, concluded that, even though ozone causes adverse health effects, no 'bright line' distinguishes one proposed standard from another in terms of protecting public health.

The regulation of airborne particulates is even more fraught with uncertainty (see *Nature* 380, 11; 1996). CASAC only endorsed a 2.5-micron standard this year after much internal argument. The EPA says its recommendations are based on reviews of 271 scientific studies. But the in-house reviews, and the subsequent outside reviews by CASAC, were rushed because of a court order forcing the agency to propose a new regulation for particulates by 29 November.

The lack of scientific consensus is already being used as ammunition by industry groups opposed to tighter regulations. The new rules, if enacted, would probably force reduced emissions from cars, factories and utility companies, and would put hundreds of municipalities in breach of air pollution laws. The public, including scientific groups, will have 60 days to comment on the EPA proposal. The regulations would not come into force for at least five years.

Industry lobbyists fired the first salvo in the debate even before the agency went public with its new standards. The Air Quality Standards Coalition said that regulatory changes are "scientifically unjustifiable," and would "produce no significant improvement in public health".

Congressional opponents are likely to sound the same theme. During the last term, Congress voted itself the power to review specific government regulations as opposed to broad laws, and the new air pollution standards will be among the first tests of this new authority. And, unlike many of the environmental debates in the last Congress, serious scientists will be weighing in on both sides of this one.

**Tony Reichhardt**

## Panel asked to rule on US-Indian patent row

**New Delhi.** The United States has asked the World Trade Organization (WTO) to set up a panel to resolve a long-standing dispute with India about patents, following the failure of the two countries to reach agreement through bilateral talks. The dispute centres on India's refusal to give patent protection to pharmaceutical and agricultural chemical products.

When it joined the trade body in 1994, India promised to protect foreign drug patents by 2005. But the United States and other countries want New Delhi to crack down sooner (see *Nature*

383, 656; 1996). The United States claims its drug companies lost as much as US\$2 billion in sales in India last year because of intellectual property piracy.

Now that the WTO's dispute settlement mechanism has been put in motion, the panel must be set up within 30 days. It will then have six months in which to make its report. In addition to the United States, the European Union has asked to be represented on the panel, as it has also been involved in bilateral talks with India on this issue.

**K. S. Jayaraman**



# Societies join forces to lobby Congress

**Washington.** The major US professional societies representing physicists, chemists and biologists are to join forces in a lobbying offensive designed to persuade Congress to increase support for basic science, and in particular for the National Science Foundation (NSF).

The move follows an awareness that energetic lobbying over the past two years by the Federation of American Scientists for Experimental Biology (FASEB) has played a substantial role in securing a budget increase of about 6 per cent in each year for the National Institutes of Health (NIH). In contrast, the NSF has seen its budget increase by only about 2 per cent.

The presidents of FASEB, the American Chemical Society (ACS), and the American Physical Society (APS) met last month to discuss a joint lobbying strategy for the budget for fiscal year 1998 (which starts next October). Afterwards they told their government relations staff in Washington to work out details of a joint approach to key congressional leaders.

Such details have not yet been agreed. But officials at each of the three groups say that they plan to make their case in Congress in time to influence the 1998 budget, proposals for which will be submitted by the Clinton administration to Congress in February, before it is split up for consideration by separate appropriations subcommittees.

The idea for the joint approach sprang from concern that the NSF has not done as well as it could have in the past two budget cycles, despite the stated view of congressional leaders that they support basic science. Additionally, society officials — especially Ron Breslow, this year's president of the ACS — feel strongly that relations between the societies ought to be improved.

The APS and ACS have not enjoyed good relations in recent years. For example, they engaged in a long, semi-public feud over the 'Science in American Life' exhibition at the Smithsonian Museum in Washington (see *Nature* **380**, 95; 1996). Both have enviously eyed FASEB's success in lobbying for funds for biomedical research at the NIH.

Last month, FASEB invited officials from APS and ACS to its annual 'consensus' meeting, at which representatives of the biology federation's member societies discussed their approach to next year's Congress.

The meeting is organized to help FASEB to agree an NIH funding 'target' which, in the past two years, has proved an accurate prediction of NIH's funding prospects. According to one FASEB official, this year the federation may also publish a 'target' for the NSF as well.

Mike Lubell, of the APS's government affairs office, says that the three groups "are

moving rather vigorously" towards an agreement. "We hope to be geared up by February," he says. The APS's physics planning committee has set up a subgroup, chaired by Pierre Hollinberg of Yale University, to develop links with other disciplines.

"The time has come for Congress to understand that science cannot be divided up into little pieces," says Lubell. He says



that targets for joint lobbying will include Bob Livingston (Republican, Louisiana), chair of the House of Representatives Appropriations Committee, and James Sensenbrenner (Republican, Wisconsin), chair of the House Science Committee.

The chemical society, with 152,000 members and an annual income of \$260 million

(mainly from publishing), is larger than the other two groups combined. Its physics counterpart has 40,000 members and an income of \$30 million, while the biology federation has an income of \$12 million, and its member societies have 43,000 members.

The ACS tends to be dominated by industrial chemists and engineers, and critics contend that it has not wielded as much influence as it could have done on science issues. Breslow, a professor of chemistry at Columbia University in New York, says that he has agreed the principle of a joint approach with the other two groups, and might invite others to join in later.

"We think that presenting the case for science together makes a lot of sense," says Breslow. "We are bigger than they are, but that's not the point: FASEB and the APS are both pretty well-connected in Washington."

John Suttie, FASEB president and a professor of biochemistry and nutrition at the University of Wisconsin at Madison, is cautious about the plan, saying that "we have not decided how to proceed to the next step". But "something will be done", he adds.

FASEB, says Suttie, acknowledges that efforts to win funding for the life sciences directorate alone at NSF are ineffective, as the directorate gets a fixed proportion of NSF's total. He thinks that a unified message will go down well in Congress: "We can't afford to have scientists squabbling," he says.

**Colin Macilwain**

## UK human genetics panel established

**London.** The United Kingdom's new Human Genetics Advisory Commission is to be chaired by Sir Colin Campbell, vice-chancellor of the University of Nottingham and former chairman of the Human Fertilization and Embryology Authority. The commission's full membership was announced on Monday (2 December) by Ian Taylor, minister for science and technology.

The 10-member commission was called for by the House of Commons Select Committee on Science and Technology in July 1995. The government initially rejected the suggestion as "unnecessary" (see *Nature* **379**, 195; 1996), but later changed its mind, accepting that such a commission might help to allay public fears of developments in genetic science.

According to Campbell, the commission will advise the government when it believes that new legislation is necessary. But its role is also "oriented to informing the public and listening to the public", and in particular to emphasizing to the government and the public where

"promise or danger" might lie in new scientific developments.

Taylor expects the commission to be "capable of anticipating fears" that the public might have about new genetic technologies, to prevent "disproportionate reactions" that might hold back successful exploitation of such research. Reports will be made public, and Campbell says he "would not rule out open meetings", although public access would not be relevant on every occasion.

The members of the commission include Martin Bobrow, professor of genetics at the University of Cambridge, the Reverend Dr John Polkinghorne, a former physicist who is also chairman of the Advisory Committee on Genetic Testing, and George Poste, chairman of research and development at SmithKline Beecham. Lay interests will be represented by Moira Stuart, a newsreader on BBC television. The secretariat will be based at the Office of Science and Technology, which will finance the commission jointly with the Department of Health.

**Claire O'Brien**



# Costs cloud Korea's synchrotron expansion

**Pohang, South Korea.** South Korea is racing ahead with plans to expand the numbers of beamlines and users for its first synchrotron, based at Pohang University of Science and Technology (Postech). But it remains unclear where all the users will come from and how the operational costs will be shared between the government, Postech and the Pohang Iron and Steel Company (Posco), which backs the project.

The Pohang Accelerator Laboratory (PAL) began routine experiments on its 2-GeV synchrotron, the Pohang Light Source, last year, using two beamlines. The successful completion of the synchrotron in 1994 was a considerable feat as, although it is Korea's first, it is an advanced third-generation machine. The number of users has reached several hundred, according to Won Namkung, director of PAL.

The synchrotron has six beamlines, including one for advanced lithography run by the LG Semicon company. PAL intends to add three new beamlines every year for the next decade with a final target of 40 beamlines and 1,500 to 2,000 users by 2008, says Namkung. The government has set aside a budget for three new beamlines in 1997 — each costing on average about US\$2 million. But it has yet to be resolved how some of the operational costs will be covered.

A large proportion of the initial costs of both Postech and PAL were paid by Posco, the world's second-largest steel-maker (after Nippon Steel of Japan), as part of the

company's effort to feed back some of its huge profits to the local community. Almost two-thirds of the \$200-million cost of the light source was paid by the company.

But Posco is beginning to distance itself from the university and PAL. It has paid its last instalment of \$150 million to the Postech foundation, which will manage an



**Common interests? The steel company that co-funds the synchrotron at Postech (above) is distancing itself.**

endowment fund for the university with an annual return of \$50 million. And the company has also withdrawn many of its researchers from the Research Institute of Science and Technology, which shares the Postech campus, and has put them back in its corporate laboratories.

The marriage of company and university research does not seem to have been a particularly happy one. The company was looking for short-term practical returns, whereas faculty members are more interested in long-term basic research.

Despite Posco's moves to withdraw from direct involvement in Postech, the steel company last week decided to establish a new

\$25-million fund to help the university with the high running costs of the synchrotron. Costs amount to more than \$20 million a year if the construction costs of the new beamlines are included.

The government is taking on a larger share of the costs of PAL and it is being operated as a national facility affiliated to

Postech for use by scientists throughout Korea. It is also being used by a few overseas scientists. But exactly how operational costs will be shared is still "under negotiation", and it will take time to reach a final decision, says Namkung.

One problem is that Postech is a private university, and it is unprecedented for taxpayers to cover the operating costs, particularly salaries, of a private institution. The university argues that

the salaries should be considered part of the operating costs of the national facility, and should be covered by the government. But the government is prepared only to pay half the \$6-million annual bill for the 150 staff at PAL, the university paying the rest. Namkung says PAL refused to agree that a users' fee be introduced to cover some of the costs.

Another challenge facing the laboratory is the recruitment of users. A Korean Synchrotron Users' Association has been set up and now supports more than 200 members, but many more users will be required to justify the laboratory's expansion.

Postech has made some attempts to recruit overseas users. An Australian synchrotron users' group that already has a beamline at Japan's Photon Factory has been courted. But at the end of last year the Australian government decided to put more money into the Photon Factory and a new collaboration with the Advanced Photon Source at Argonne National Laboratory in the United States. The Argonne machine is more powerful than Korea's (see *Nature* 378, 653; 1995).

There is a handful of users from Japan and Taiwan. But the vast majority are Korean. At a meeting in Seoul earlier this month, science ministers of the Asia-Pacific Economic Cooperation (APEC) forum called for greater sharing of national research facilities in the region. Facilities at Postech were put forward by the Korean government for use by APEC scientists under the forum's planned Advanced Science and Technology Network (see *Nature* 384, 200; 1996).

But Namkung says that foreign scientists "should not dominate the users' time" and PAL may "impose a cap or designate specific beamlines for international use. Others go further, and suggest — informally — that Korea should not be expected to support the research of scientists from developed countries.

**David Swinbanks**

## India approves genetic diversity study

**New Delhi.** The Indian government has approved a five-year, US\$20-million project to study the genetic variations of India's diverse population groups. The decision comes after much debate on ethics and research priorities.

The programme, described as the national version of the global Human Genome Diversity Project, will be run by the Department of Biotechnology. "We are giving this high priority," says Manju Sharma, secretary of the department.

One goal of the genome project is to understand how Indian communities differ genetically from one another and from peoples in other parts of the world. Another is to understand the relationship between genomic variation and disease patterns in the groups studied. India has 465 communities, including 75 endangered tribal groups.

According to the biotechnology department, the project may give priority to vanishing tribes in the Andaman islands, groups in Kerala state with distinctive

features, and India's 300,000 Parsees, who experience a high incidence of breast cancer. Four laboratories are being dedicated to this project and a national repository of biological samples, such as DNA and cell lines, is going to be enlarged, says Sharma.

The department's guidelines for the project specify that blood can be drawn only from volunteers, and communities must share any benefits that accrue from the investigations. Funds will be available on condition that research is done by Indian scientists in Indian laboratories, and that no samples are sent out of the country.

Government officials admit that blood samples and DNA have in the past been sent abroad without the knowledge of Indian agencies (see *Nature* 379, 381; 1996). Sharma says that he hopes that this practice will now stop as "we are going to fund this research and create the facilities required for genomic analysis in India itself".

**K. S. Jayaraman**

# Warning of 'research crisis' at German universities

**Munich.** Germany's independent science advisory council, the Wissenschaftsrat, warned last week that the quality of research in German universities is facing a crisis because of reduced funding, inflexible attitudes and increasing teaching burdens.

The Wissenschaftsrat says that urgent measures are needed to safeguard universities' research potential if the traditional 'Humboldt principle', which emphasizes the need to link teaching and research, is to be maintained. The warning is delivered in a series of ten 'theses' — a traditional means of expression in academic circles that dates back to Martin Luther.

In a message directed mainly at federal and regional politicians, the council says that more investment is needed to modernize university laboratories, which, in nearly all disciplines, have become increasingly neglected since the expansion of universities that began in the 1960s.

Student numbers in Germany have almost doubled since 1980. Yet there has been no growth in university budgets, or in the number of academic staff. Greater student numbers not only force academic staff to spend more time teaching but also divert investment away from laboratories. The situation has now reached crisis point, according to the Wissenschaftsrat.

As a result, Germany's most talented researchers generally prefer to work in better equipped non-university research institutes, such as those of the Max Planck Society. This is particularly true in east Germany, where university research was discouraged in communist times, and also in high-technology disciplines in which equipment needs to be continually updated.

But money alone is not the answer, says the Wissenschaftsrat. Its theses also urge universities to shed inflexible attitudes that blind them to the fact that modern research is increasingly interdisciplinary and international.

The high degree of independence given

to faculties is partly to blame, it says, because it encourages competition between faculties, rather than cooperation in establishing interdisciplinary research programmes. Universities should avoid internal squabbling and focus their energies on competing in a more global sense.

Research sabbaticals should not be an automatic privilege but should be awarded on merit, and a university's budget should be distributed on merit, rather than being divided fairly evenly between disciplines.

The council also says that university research must acknowledge new social demands, more positions must be found for young woman scientists, and university scientists should become more involved in public debates on the acceptability of certain areas of research.

The University Rectors' Conference (HRK) has welcomed the theses, which mirror its own long-standing concerns about standards of university research laboratories. Josef Langer, general secretary of HRK, says that the situation is deteriorating, with cuts by *Land* (regional) governments to their universities averaging well over 5 per cent this year.

But not everyone feels that tight budgets are undermining the quality of research. Christoph Schneider, spokesman for the Deutsche Forschungsgemeinschaft (DFG), the organization that distributes competitive research money to universities, says that the research body "sees too many good research proposals passing through [the DFG] to recognize an impending danger for university research".

Nevertheless, DFG accepts that it is difficult to encourage talented scientists to return to Germany after postdoctoral experience in the United States. It has recently launched a 'young scientist programme', which will pay for young biologists who are deemed to be excellent to return to Germany and set up their own research groups in universities.

**Alison Abbott**

## Illegal sale of research ship prompts review

**Moscow.** The presidium of the Russian Academy of Sciences has been reviewing the future of its research fleet after one of the vessels, *Hydrobiolog*, was sold off without permission. The ship was leased by its owner, the P. Shershov Oceanology Institute, to a private company for gathering coral off the coast of Tunisia. But the businessmen registered the vessel in Tunisia, then changed its name and finally sold it illegally.

Nikolai Laverov, vice-president of the academy, says that the academy allowed

the ship to fly the Tunisian flag to avoid paying taxes, because otherwise the leasing would have been uneconomic. But the businessmen took advantage of this permission and fraudulently sold the ship.

The academy itself has been keen to sell off some of its old ships to support the newer ones, but at present this is impossible as all the vessels are part of the scientific research fleet. The academy is going to withdraw this status from the *Zarya*, *Aquanavi* and *Aquanavi-2*.

Carl Levitin

## Engineering academy agrees compensation for deposed president

**Washington.** The US National Academy of Engineering (NAE) has agreed to pay \$687,500 to Harold Liebowitz, who was recently deposed as president, as compensation for his removal from office one year into a four-year term.

The academy, whose main activity is to provide the US government with advice on technical problems, also expects to incur legal costs of \$300,000, bringing the total cost of Liebowitz's departure to almost \$1 million.

Liebowitz, a former home secretary of the academy, stood for president twice as a 'rebel' candidate running against the established order in the NAE. He complained that the academy was neglecting its membership of 2,000 élite engineers, and failing to assert itself relative to its larger and more powerful scientific counterpart, the National Academy of Sciences (NAS).

He won the second time, in April 1995, but soon clashed with staff at the National Research Council — the operating arm of the two academies — as well as with senior officials at both NAE and NAS. In May, the council of the NAE won backing from the



**Liebowitz: voted out of office by NAE members.**

membership for a rule change that enabled the membership to vote Liebowitz out of office (see *Nature* **380**, 471; 1996).

William Wulf, acting president of NAE until new elections are held, says the agreement with Liebowitz will bring an end to the episode. But he says it has taught the academy to pay more attention to its members.

"I don't think there is any question that the academy needs to be more responsive to its membership, and that is what we are now doing," says Wulf. Liebowitz, who has withdrawn all of his various legal actions against the academy complex and its officers in exchange for the settlement, was unavailable for comment.

An undisclosed portion of the settlement will be paid by the NAE's insurance. Some of the rest will come out of overhead charges on the academy complex, and up to \$325,000 from the NAE's endowment funds, which is currently worth \$40 million.

Wulf justifies making Liebowitz's payment out of the money paid by the US government as overhead costs on federal contracts on the grounds that "legal fees are part of the natural cost of doing business".

**Colin Macilwain**



# French cash pull-out puts Cluster in doubt

**Munich.** New doubts have been raised about whether the Cluster satellite mission will fly again, despite support for the plan from the European Space Agency (ESA). Last week, France unexpectedly told ESA's decision-making science programme committee (SPC) that it is not prepared to pay for the scientific instruments, which were destroyed when Cluster's Ariane-5 launcher exploded during its first launch in June, to be rebuilt.

France is one of the biggest participants in the four-satellite Cluster programme. If it does not reverse its decision, the relaunch, known as Cluster II, will almost certainly have to be abandoned. Under ESA's standard procedures, Cluster's four satellites must be paid for by ESA. But the scientific instruments that the satellites carry as payload must be paid for by participating member states.

Last month, ESA's space science advisory committee voted unanimously to support Cluster II, backing a ECU210-million (US\$168-million) plan to build the four new satellites and pay for their launch (see *Nature* 384, 99; 1996).

Even the day before last week's SPC meeting, participating member states — including the United States, the United Kingdom, France, Germany and Sweden — were said to be optimistic that money would somehow be made available for instruments. Although they are still very expensive, the cost of replacing the instruments is less than

half the original cost, which included all the development expenses. The cost of rebuilding the instruments, according to a 'minimum payload plan' designed by the Cluster programme committee, would be around £7 million (US\$4.16 million) for the United Kingdom, for example, less than DM10 million (US\$6.53 million) for Germany, and around FFr50 million

have been given by either the French research ministry or CNES, the French space agency. ESA officials declined to comment on Monday, but a statement from d'Aubert's office indicated that he might be prepared to negotiate with ESA over payment for instruments "once he has more information".

One factor in France's sudden reluctance to finance Cluster II could be the future of Ariane-5. This is part of ESA's optional launcher programme, which is where France has its greatest interest. The Ariane-5 programme found itself short of hundreds of millions of ECU as a result of the failure of its maiden voyage (see *Nature* 383, 368; 1996). France may prefer any available funding to be used in support of the launcher, rather than in saving a basic science mission.

Despite the uncertainty surrounding the financing of the payload, the SPC voted to continue planning for Cluster II. It will put the building of the satellites out to tender in the next few weeks. But a final decision on whether Cluster II will be built will have to wait for SPC's next meeting in February.

Meanwhile, both ESA's space science directorate and Cluster scientists are hoping that d'Aubert will be persuaded to change his mind. Paul Murdin, SPC delegate from Britain's Particle Physics and Astronomy Research Council, admits that the French announcement is "obviously very difficult", but says he remains optimistic that Cluster II will go ahead.

**Alison Abbott**

IMAGE  
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REASONS

**Cluster's last stand? French priorities may lie with Ariane-5 rather than Cluster (above).**

(US\$9.57 million) for France.

But only a few hours before the SPC meeting began, the French delegation received instructions from François d'Aubert, France's research minister, to inform ESA that France would not contribute anything to the new payload plan. No explanation for the unexpected announcement appears to

## Policy differences 'threaten Israel's biotech potential'

Jerusalem. Disagreement within the Israeli government about the economic potential of the country's fledgling biotechnology industry could prevent the sector from reaching its full potential. That was the message from speakers last week at a conference at the Weizmann Institute of Science in Rehovot sponsored by the National Committee for Biotechnology.

Haim Aviv, chairman of the committee and a former professor of biology at the Weizmann Institute, said that the industry could improve on its 1995 sales of US\$249.3 million by an order of magnitude over the next ten years. But it needs investment of between \$100 million and \$200 million to do so. Such investment is unlikely to come from private-venture capitalists, said Aviv, as the industry is still in its infancy and there are long lead times between basic research and marketable products.

The Ministry of Science seems to share the confidence that Israel has the potential to become a major player in biotechnology. It has budgeted almost \$6 million this year for development projects intended to bridge the gap between basic research and market-

able products. But the Department of Commerce is holding back from making any major commitment, saying that it is unconvinced that Israel has a competitive advantage in biotechnology.

Leah Boehm, chief scientist at the science ministry, pointed out that more than one third of Israel's scientists work in the life sciences. Aviv confirmed this potential by adding that one per cent of all publications worldwide in the life sciences are by Israeli authors — 100 times more than would be expected from the number of scientists in the country.

The problem, Aviv said, is that Israel has not been good at translating its basic research into products. But he believes that is changing. He pointed out that Copaxone, a multiple sclerosis drug developed by Israel's largest pharmaceutical company, Teva, has won approval this year from the US Food and Drug Administration.

But the commerce department, which has a much larger budget than the science ministry for supporting research and development in new industries, is cooler about the economic potential of biotech-

nology. This is despite the fact that it approved this year a \$49.2-million, five-year investment fund for the development of drugs and diagnostic tools, and a similar \$7.5-million grant for the development of seaweed products.

Yishai Laks, adviser to the commerce department's director-general, says: "We don't believe that the biotechnology industry is an appropriate place to make massive investments. It has only long-term potential and we are not convinced that Israel has a competitive advantage. If the private sector has avoided investing in the field, we shouldn't [invest] either."

According to Laks, the department believes that Israel will benefit more from government investment in high-tech electronics. But Aviv argues that it is precisely because biotechnology is still too risky to attract private investors that the government needs to make a major commitment. One of the reasons that Israel has been so successful in electronics, he claims, is that the defence ministry invested heavily in the field and created the infrastructure that biotechnology lacks.

Haim Watzman

# Novel pathogens beat food safety checks

**Paris.** Renewed calls were heard last week for regulatory systems to adapt to the threat of novel pathogens in the food supply after pies and gravy contaminated with the 0157:H7 strain of *Escherichia coli* killed five people in Scotland.

The outbreak of food poisoning — the worst in the country's history, infecting 260 people — is the latest in a series caused worldwide by the 0157:H7 strain since it was first detected in 1982. More than 600 people were affected in a US outbreak in 1987, while in Japan around 10,000 people were infected in an outbreak earlier this year that left 11 dead (see *Nature* 382, 567; 1996).

The bacterium produces powerful cytotoxins that can cause bloody diarrhoea (haemorrhagic colitis), haemolytic uraemic syndrome (HUS) and kidney failure. Mortality among those infected can be 5 to 10 per cent.

Clusters of such cases reported in the literature in the 1940s and 1950s in the United States and Europe closely resemble contemporary 0157:H7 outbreaks. This suggests the strain was present long before it was identified in 1982, says Bernard Rowe, head of the UK Public Health Laboratory Service's Laboratory of Enteric Pathogens.

The almost identical sequence of the toxin genes of the *E. coli* strain with those of the human pathogen *Shigella dysenteriae* Type 1 is widely considered to show that 0157:H7 acquired the genes from *Shigella* through DNA swapping, says Mike Hudson, a specialist on the strain at the UK Centre for Applied Microbiology and Research at Porton Down in Wiltshire.

Hudson sees an increase in new food pathogens, including *E. coli* 0111, another toxin-producing strain, and strains of *Salmonella enterica* resistant to temperatures that kill other bacteria during food processing. Antibiotic resistance has also become common. "We need to come to grips with how new pathogens appear in the food supply," says Paul Neal, an official at the food-borne diseases division of the US Center for Disease Control in Atlanta, Georgia.

Gene transfer in the wild is poorly understood, as is the impact of environmental changes on such processes, says Hudson. He speculates that the appearance of some new strains may be linked to changes in farming practices. Lack of understanding of the impact of changes in food production on microbial ecology means that "we could be storing up problems for ourselves", he says.

Some scientists are more sanguine, however. Christopher Higgins, professor of clinical biology at the University of Oxford, says he is not convinced that novel food pathogens are on the increase. Observations of so-called novel food poisoning agents

may be largely accounted for by improvements in the reporting of cases of illness, he argues. He says that changes such as the increasing use of frozen foods simply demand that appropriate food safety measures be introduced.

Many scientists argue, however, that the increasing industrialization of food production is a double-edged sword. They say that, although it has allowed major improvements

in quality control, the increase in the scale of operations now means that "when something goes wrong it goes wrong in a big way".

Last week's food poisoning outbreak was centred on the town of Wishaw in Lanarkshire, southwest Scotland, and has been traced to pies and gravy produced by John Barr, a butcher and beef wholesaler who was recently nominated 'Scottish beef butcher of the year'. The British government immediately launched an inquiry into how the contamination occurred, and its implications for food safety procedures.

The frequency of outbreaks of 0157:H7 throughout the world has steadily increased from 20 in 1983 to more than 800 last year. This is probably because people are now looking for it more carefully. But there is still evidence of "a slow but steady rise", according to Rowe.

He believes 0157:H7 will not reach the epidemic proportions of *Salmonella enterica*, which causes tens of thousands of cases annually in the United Kingdom alone. This is because the *E. coli* strain only occurs in around 1 per cent of cattle while *Salmonella* is widespread in poultry.

But Rowe acknowledges that the strain "is a very severe infection that has to be taken extremely seriously". Fewer than a dozen bacteria can be enough to cause illness. Most common food pathogens, such as *Salmonella*, cause illness only when they are present in food in thousands.

This low infectious dose means that control measures will need to eliminate 0157:H7 almost completely from the food chain. This makes it "the ultimate test for the regulatory system", says Hugh Pennington of Aberdeen University, the chairman of

the UK inquiry panel. "It is testing the regulatory system and the system is failing."

"The things we are discovering about 0157:H7 are demanding us to rethink some of the fundamentals by which we control food safety," adds Catherine Reynolds, spokeswoman for the UK Institute of Food Research. The strain can survive acid conditions, for example, by becoming dormant until conditions become more suitable.

There is an urgent need for better understanding of the survival mechanisms of bacteria under non-growing conditions, says Reynolds.

The ability of 0157:H7 to survive acid environments increases the risk of foods such as mayonnaise and salami becoming infected.

The main reservoir of 0157:H7 is the gut of cattle. Its main route into the food chain is through contamination of meat by intestinal contents and faeces in the abattoir. The organism cannot be eradicated from cattle as it is now part of their normal flora, says Rowe. "We have got to tighten up the food chain absolutely everywhere".

Most outbreaks have been linked to hamburgers or minced meat products. Mincing results in bacteria being transferred to the interior of such products where they can survive all but thorough cooking. With steaks and other meat cuts, in contrast, bacteria are restricted to the outer surface where they are readily killed by cooking. But infections have also occurred through milk, fruit juices, water and person-to-person contact. "We are recognizing a growing role for fruits and vegetables in outbreaks in the United States over the past two years," says Neal.

The United States has already tightened procedures in response to the outbreak in 1987, introducing a 'pathogen reduction programme' throughout the meat industry. This represents a major shift in quality control procedures, according to Neal, away from random inspection of individual carcasses to monitoring general microbial levels throughout the chain and taking steps to reduce them. "We are trying to determine whether the process is minimizing contamination at every step," says Neal. He adds that, by reducing overall microbial levels, the risk of 0157:H7 contamination should also fall.

The appearance of the strain has strengthened calls from consumer associations in the wake of the 'mad cow' crisis in Europe for systems that allow food products to be traced all along the food chain "from the farm to the table".

Tracking the origin of foods in outbreaks of food poisoning is notoriously difficult. "We can identify the food fairly easily, but working back to the animals is horrendously difficult, and the track often runs cold," says Pennington.

**Declan Butler**

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**Food for thought: passers-by ponder the shop window of the closed butchers where the latest *E. coli* outbreak originated.**



# German lab ordered to tighten monitoring

**Munich.** One of Germany's three former national nuclear research laboratories has come under fire from the Öko Institute, a leading environmental research organization, for failing to keep adequate track of some of its nuclear materials.

An unpublished report from the institute — a long-standing critic of nuclear energy — says that the GKSS (Forschungszentrum Geesthacht), near Hamburg, cannot be ruled out as the cause of a recent cluster of childhood leukaemias in its vicinity.

In addition, a tightening of the laboratory's monitoring and administrative procedures has been ordered by the energy ministry of Schleswig-Holstein, the *Land* (state) in which GKSS is located and which is run by the Social Democratic Party.

The report was commissioned by the *Land* last year. Although it has not yet been officially published, its allegations of lax supervision were leaked to the press last week, and have stirred up the German public's traditional sensitivity to nuclear issues.

The Krümmel nuclear power plant, adjacent to GKSS, was initially suspected as the cause of the nine cases of leukaemia in neighbouring villages since 1989. But a report two years ago from the institute found no evidence of either increases in waste output from the plant, or leaks of radiation, which could account for the cluster.

It was after this that the latest report on GKSS, covering the period 1982 to 1993,

was commissioned. The authors say that they found no direct evidence that leaks from GKSS might have been responsible for the leukaemia cases. But they say that they did find serious deficiencies in the centre's monitoring of its nuclear activities, making it impossible to draw firm conclusions on a possible relationship between GKSS and the cancer cluster.

GKSS was set up in 1956 as one of three national research centres in Germany concerned with the development of nuclear power. Despite a radical change in its orientation towards environmental research in the past few years (see *Nature* 370, 7; 1994), the centre still works with several sources of nuclear material.

One is an active research reactor, used for creating a variety of radioactive isotopes. According to the Öko Institute's report, this has always been well monitored, and can be ruled out as a possible cause of the cluster. In contrast, it says, the fate of some of the isotopes themselves, which are distributed to research institutes all over northern Germany, has not been so strictly monitored.

Tens of thousands of probes, containing elements such as thorium-232, plutonium-238, strontium-90 and radium-226, were produced during the 12 years covered by the report. Records of 63 of these are missing.

GKSS also acts as an interim store for several hundred containers of nuclear waste from hospitals, universities and

research institutes in northern Germany.

The report criticizes the lack of regular checks on the integrity of the containers, and the absence of alpha- and beta-radiation detecting equipment in neighbouring villages. It says such equipment should have been installed to provide regular checks on the exposure of the local population.

According to one of the report's co-authors, Christian Küppers, a physician, "levels of radiation relevant to leukaemia could have escaped" if there had been any fire in, or leak from, any of the containers.

Andreas Fleck, an official of the ministry of health, which is responsible for the nuclear storage site, admits that the containers are not examined regularly. But he insists that any serious radiation incident would have been detected by equipment in the depot housing the containers.

GKSS itself is declining to comment on the allegations of poor record-keeping until the Öko Institute's report is published. But officials of Schleswig-Holstein's ministry of energy say that, in response to the report, they plan to request immediate improvements in GKSS's administrative and organizational activities.

Such improvements include reliable monthly documentation of all the centre's activities involving nuclear material, installation of equipment at the research reactor to provide direct and accurate measurements of alpha-radiation. **Quirin Schiermeier**

## Oxford and Cambridge still dominate UK citations list

**London.** Oxford and Cambridge Universities continue to dominate the rankings of British universities in a new survey of science citations. But there is a difference in emphasis between the two: Cambridge has the highest number of citations for papers in physical and chemical fields, while Oxford takes the lead in biological subjects.

The study, by the Institute of Scientific Information (ISI) in Philadelphia, covers scientific papers published and cited between 1991 and 1995 in ISI-indexed journals. Winning topics for Cambridge are physics, chemistry, materials science, engineering, geosciences, astrophysics and maths.

Oxford claims second place for citation numbers in all these subjects, and first place for immunology, microbiology, biology and biochemistry, molecular biology, ecology/environmental science and economics/business. Cambridge comes either second or third in five of these areas.

The Oxbridge domination is "not unexpected", according to an article in the January issue of the ISI's newsletter, *Science Watch*, because the citation ranking favours large institutions that produce many papers.

Other strong performers were University

| Field                     | 1                           | 2                           | 3                            |
|---------------------------|-----------------------------|-----------------------------|------------------------------|
| Physics                   | Cambridge (14,801)          | Oxford (10,567)             | Imperial Coll. (7,877)       |
| Chemistry                 | Cambridge (13,881)          | Oxford (7,908)              | Imperial Coll. (4,813)       |
| Materials science         | Cambridge (1,753)           | Oxford (973)                | Imperial Coll. (846)         |
| Engineering               | Cambridge (2,357)           | Oxford (2,088)              | Imperial Coll. (2,008)       |
| Geosciences               | Cambridge (2,993)           | Oxford (2,292)              | Edinburgh (1,558)            |
| Astrophysics              | Cambridge (8,669)           | Oxford (3,875)              | Leicester (2,392)            |
| Computer science          | Edinburgh (154)             | Imperial Coll. (98)         | York (93)                    |
| Mathematics               | Cambridge (794)             | Oxford (634)                | Imperial Coll. (467)         |
| Ecology/environmental     | Oxford (1,314)              | Imperial Coll. (1,299)      | Lancaster (864)              |
| Agricultural sciences     | Reading (847)               | East Anglia (617)           | Aberdeen (588)               |
| Plant and animal sciences | East Anglia (3,570)         | Oxford (2,212)              | Bristol (2,209)              |
| Clinical medicine         | Univ. Coll. London (20,195) | Oxford (19,040)             | King's Coll. London (12,897) |
| Immunology                | Oxford (7,524)              | Univ. Coll. London (3,590)  | Cambridge (3,188)            |
| Microbiology              | Oxford (3,594)              | Cambridge (2,554)           | East Anglia (2,157)          |
| Biology and Biochemistry  | Oxford (20,088)             | Univ. Coll. London (14,530) | Cambridge (13,746)           |
| Molecular Biology         | Oxford (19,924)             | Cambridge (14,269)          | Univ. Coll. London (13,834)  |
| Neuroscience              | Univ. Coll. London (10,635) | Oxford (8,376)              | King's Coll. London (6,499)  |
| Pharmacology              | King's Coll. London (2,440) | Univ. Coll. London (2,251)  | Cambridge (1,986)            |

College, London, with first places in neuroscience and clinical medicine, and King's College, London, which heads the list in pharmacology and psychology/psychiatry. Imperial College, London, achieved second or third rankings in seven fields, principally the physical and mathematical sciences.

But a separate ranking of British universi-

ties by citations per paper, where departmental size makes less impact, produced a different picture. Here the top score went to molecular biology at the University of Dundee, with an average of 17.74 citations per paper. The University of Durham came top in astrophysics, and the Open University in geosciences. **Claire O'Brien**

## NSF seeks to sharpen up process of peer review

**Washington.** The National Science Foundation (NSF) is to ask its reviewers to grade the 'broader impact' of each research proposal, as well as its intellectual merit, under new rules which it is considering for implementation next year. If the changes go ahead, reviewers would grade each proposal from 'poor' to 'excellent' under both of these criteria, and to comment on both criteria.

At present, reviewers give each proposal just one grade and one commentary. The grade is supposed to be based on a list of four detailed criteria: competence, intrinsic merit, utility and impact on the US science infrastructure. But NSF surveys have indicated that reviewers find these criteria verbose, and often ignore the last two.

Neal Lane, director of the NSF, says that the changes "will encourage reviewers to comment on more aspects of the proposal". He says that the objective is to provide better information to the programme officers who make final decisions on NSF grants, based on the reviews, and not to change the type of work funded. The NSF solicits 170,000 reviews each year of the 30,000 grant proposals it receives. □

## FDA chief plans to quit

**Washington.** David Kessler, the paediatrician and lawyer whose controversial tenure as head of the US Food and Drug Administration (FDA) earned him the admiration of public-health advocates and the enmity of industry, announced last week that he is to resign after six years at the helm of the agency. Kessler will step down once a new agency chief is named, which is expected to be early next year.

Internal FDA candidates include two deputy commissioners, Mary Pendergast and William Schultz. But some Republicans in Congress insist that an outside candidate is needed to change the culture of the agency. Kessler expanded the agency's reach, tightening pre-market review standards for medical devices, and putting new limits on cigarette advertising to children. He also obtained the drug industry's support for the Prescription Drug User Fee Act, which has improved approval times for cancer and AIDS drugs. □

## Call for global energy reforms

**London.** The World Energy Council, a nongovernmental, noncommercial organization representing energy interests from more than a hundred countries, called last week for a global effort led by the industrialized countries to forge a "pathway to sustainable development" by reconciling economic and social development based on increased energy use with protection of the environment.

The council says that action is required now in such areas as raising global energy efficiency, charging full market prices for energy, improving confidence in nuclear power, curbing atmospheric emissions and extending the principle that 'the polluter pays'. It also points out that by 2050, developing countries are likely to be using more than 60 per cent of primary energy supplies, and contributing the greatest proportion of atmospheric emissions. □

## German budget cuts approved

**Munich.** The sun seems to have stopped shining on the German research minister, Jürgen Rüttgers. As part of the 1997 budget, approved by the German parliament last week, his ministry will receive only DM14.8 billion (US\$10 billion) next year, 5.6 per cent less than this year. This cut is more than double the average cut to ministries of 2.5 per cent.

In the parliamentary debate on the budget, whose size has been known for several months, Rüttgers said that "those who have less money must use it more intelligently". To that end, he is planning a series of competitions, along the lines of the recent Bioregio experiment, which challenged academics and industry together to come up

with ideas for developing biotechnology in their regions. The three winners gained preferential access to part of the ministry's existing biotechnology budget (see *Nature* 384, 298; 1996). Rüttgers says similar competitions could help to develop medium-term research strategies in areas such as molecular medicine and new materials. □

## Data access 'open to abuse'

**London.** An inquiry by a UK House of Lords select committee into the effectiveness of European Union rules on freedom of access to information on the environment, contained in a 1990 directive, has found that they are unclear and open to abuse. The report, published on Monday (2 December), says that the directive — and thus the UK government's own regulations in the field — need amending. Clearer definitions of 'environmental information' and 'responsibilities relating to the environment' are needed, for example. □

## European plans for patents

**Paris.** The European Commission last week adopted a 'plan of action' to encourage innovation in Europe, proposed by Edith Cresson, the research commissioner, Martin Bangemann, the industry commissioner, and Christos Papoutsis, the commissioner for small and medium sized enterprises (SMEs). The plan contains, in particular, a reform of Europe's patent regulations, which will be proposed in detail in a green paper next year. Cresson complained last week that the cost of filing and maintaining a patent in eight member states costs ECU156,000, compared with just ECU17,000 in the United States. The costs and complexity of the system explained why two-thirds of Europe's SMEs held no patents, she argued. □

## Spread of HIV gains pace

**Geneva.** The AIDS epidemic is still in its infancy, according to a report published last week from the United Nations Programme on HIV/AIDS (UNAids), which warns that the virus is spreading increasingly rapidly throughout Africa and Asia and into previously uninfected countries in Central and Eastern Europe.

The report points out that sub-Saharan Africa is the most seriously affected region, where more than 1 in 20 people carry the virus. Complacency in the industrialized world over the threat of AIDS is criticized by UNAids director Peter Piot, who says that prevention of infection is the only hope for the whole world as the virus spreads into new areas and cases continue to multiply in others. This year, three million people have been infected with HIV and 1.5 million have died of AIDS. □

## Tackling antibiotic resistance

**London.** The World Health Organization and the International Federation of Pharmaceutical Manufacturers Associations have agreed to collaborate on containing the spread of antibiotic-resistant bacteria. The partnership should help research and development into new antibiotics and cost-effective treatment of infections. WHO has established a global network for monitoring antibiotic resistance, and new funding will allow specific training to be provided in laboratories in China, Indonesia, Kenya, Mongolia, Thailand and Vietnam. African laboratories are already participating in the network. □

## Targets for renewable energy

**Brussels.** Clear, ambitious and realistic targets to increase the contribution of renewable energy sources such as wind, solar, biomass and small-scale hydropower, to the energy balance of the European Union have been called for by a Green Paper published last week by the European Commission. At present, such sources only account for six per cent of energy consumption. Increasing this level would have a range of beneficial effects, including a significant reduction on carbon dioxide emissions, says the commission, which plans to publish a comprehensive strategy for renewable energy next year. □

## A case for instant peer review?

SIR — I should like to comment on your News story<sup>1</sup> arising from a recent meeting at the Royal Society and on an earlier leading article about peer review<sup>2</sup>.

My colleagues and I have repeatedly praised McKay *et al.*<sup>3</sup> for their courage in reporting the possibility of fossil life in a martian meteorite. My group's scientific record is documented by *Nature*<sup>4-6</sup>, so there was never any necessity to use the meeting "Searching for Life in the Solar System and Beyond" (SLSSB) as a forum to make any personal statement claiming precedence; in fact the meeting was the brainchild of Dr Alan Penny of the Central Laboratory for the Research Councils and was 40% devoted to searching for life around stars other than the Sun, in addition to discussing issues concerning Mars.

Further, I would like to correct a number of errors in the News story: (1) no press release containing data from anyone attending SLSSB was issued before 1250 hours on 31 October; (2) Ian Wright's very responsible comments to the press concerning Wright *et al.*<sup>4</sup> were that "our data do not show one way or the other" — rather different from discounting explicitly the possibility of life on Mars; and (3) no journalist was given an exclusive interview by anyone; indeed, *Nature* was offered the same opportunity as a number of other journalists — a personal invitation to attend the scientific sessions of SLSSB.

*Nature* is entitled to adopt any policy it wants on scientists talking to the press<sup>2</sup>. As Gresham Professor of Astronomy, I want to encourage scientific understanding of "new learning" as Sir Thomas Gresham would have wished 400 years ago when he gave money for public awareness activities. How can scientists expect the public to become interested in our work if we continue to tell them the issues are too complex for them to understand?

Finally, I believe that I, my co-authors and everyone involved in SLSSB have behaved impeccably. The media blackout before Wright stood up before his peers was totally effective. We offered ourselves on 31 October, as Ian Taylor MP, Minister for Science and Technology, so nicely put it, "for instant peer review".

**C. T. Pillinger**

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SIR — A recent leading article<sup>2</sup> criticizes the use of the popular media as outlets for scientific information and states categorically that publication in peer-reviewed journal is an essential prerequisite for public discussion.

Strict pre-publication peer-review procedures as applied by *Nature* and other scientific journals are a relatively new phenomenon in the history of science. Most of this history witnessed publication of major works (Copernicus, Darwin) without strong reliance on peer review, followed by public discussion in print by peers. In this way the entire process of the exchange of scientific arguments was transparent and allowed to bring science closer to the public.

Today, peer review suppresses debates in print, forces them to take place anonymously through editorial offices and produces publications containing compromise statements. It emasculates scientific debates and hinders publication of non-consensus views.

Negative aspects of peer review have been well recognized. David Goodstein recently stated<sup>7</sup> that the process of peer review encourages what he calls "scientific misconduct" as "referees are never called to account for what they write in their reviews". Because referees often have a conflict of interest with authors, it is naive to believe that in their anonymity they will remain meticulously impartial.

Even at the beginning of the century, it was editors of scientific journal who made decisions about publication of submitted manuscripts. As specialization increased, editors sought the advice of experts in rapidly narrowing fields but still made their own decisions. Finally it has been discovered that a major portion of editorial decision can be *de facto* made by referees.

With peer review stifling scientific communication, it is no wonder that scientists, being mostly creative people, are seeking alternative ways of making their findings and interpretations public. Scientific journals are, after all, a part of the 'media industry' selling information. So you are subject to the normal rules of a competitive market.

In the era of worldwide journalism and Internet communication, standard ways of disseminating scientific information are becoming too cumbersome, and no amount of pleading and threats to authors who talk to the media before subjecting themselves to a delaying quagmire of peer review will prevent the change.

Science thrives on unencumbered flow of information and exchange of arguments and will naturally use new opportunities in

this regard. I am sure that *Nature* will respond creatively to the challenges of the market and maintain its present role of a leading purveyor of scientific information.

**Maciej Henneberg**

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Australia

## Cistron has guts

SIR — I read with disappointment your leading article "Peer review and the courts" (*Nature* 384, 1; 1996). You denigrate Cistron Biotechnology for being a small company, and reveal your misunderstanding of the importance of IL-1 technology.

As you should be aware, IL-1 is the initiator of the human immune response. IL-1 has been a vital tool in the discovery and development of promising therapies such as IL-1 receptors and protease inhibitors and in apoptosis studies. The fact that Immunex Corporation tested the 'mature' fragment, a fragment that elicits a high level of biological activity, and found it to be toxic in clinical trials is not surprising. Other IL-1 fragments of mutants, as well as the precursor form of IL-1 itself, may well prove to be therapeutically useful.

Cistron may be a small company, but it is a publicly held company, and our shareholders would take a dim view of your suggestion that the proceeds of the litigation settlement be divided among our employees and that we take "early retirement".

I would expect better of *Nature*, especially since our lawsuit accused Immunex of stealing our IL-1 sequence from an unpublished manuscript submitted to your journal. At least Cistron had the guts to stand up for the peer-review system.

**Bruce C. Galton**

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## Prior publication

SIR — When you wrote about the report, in "50 years ago", on locating radium-labelled click beetles (*Agriotes* sp.) with a Geiger-Müller counter (*Nature* 17 October 1996, ix), you might have mentioned a method described many years earlier by J. M. Barrie, whereby the sound of ticking served to localize a crocodile that had ingested an 8-day alarm clock.

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## Japanese grant system is a lottery

SIR — The News article "Bias alleged in Japanese university awards" (*Nature* 383, 369; 1996) provides an interesting insight into some of the problems associated with Japan's university grant system. But the problems are by no means confined to the particular type of grant described in the article. Rather they are part of much bigger problems that are deep rooted in the Japanese grant system as a whole.

I recently moved from the University of Alberta, Edmonton, Canada, to Nagoya City University. During my eight years as a professor of medicine in Canada, I participated in the Canadian grant system, through the Medical Research Council of Canada (MRC) and the Heart and Stroke Foundation of Canada. I also served on one of the study sections for grant screening of the US National Institutes of Health (NIH) for four years. Thus I am in a position to comment on both the Japanese and North American systems.

There are two very important differences between the two systems. The first concerns the very concept of a research grant. To a North American scientist, the research grant is an essential part of the resources needed to carry out his/her entire research activity, including the salaries of full-time laboratory staff.

Researchers are trained how to write applications, how the reviewing system works and how they should respond to reviewers. Most research grants are renewable over a period of a few years and they become a reliable source of support for research projects.

Although, as a result of severe budget cutbacks in both Canada and the United States in recent years, there are insufficient funds to be awarded, the competitive research grant is still the key source of support for research.

In the Japanese system, a grant is viewed very differently. It is basically considered as a supplementary 'subsidy' in addition to guaranteed funds from the government that cover salaries and provide very limited research and operating funds. For the 'average' Japanese scientist, competitive grants are not a reliable source of support for their research because most of them are not renewable and last only one or two years.

Furthermore, for the vast majority of grants, the size of a single grant is generally so small that scientists must gather between five and ten different grants of a few million yen each (a few tens of thousands of dollars or less) to have comparable funds to a single Canadian or US

operating grant. This must be done every year. It must also be borne in mind that costs of research in Japan are almost double those of North America.

For most types of grants (the grants described in the news article are an exception), it is impossible to hire full-time staff with the money, both because of government regulations covering the grants and because of their unreliable nature as a source of funds.

A second important difference lies in the system for reviewing and selecting grant recipients. There is no real equivalent in Japan of the study sections of grant-awarding agencies in the West, except in the case of a few very prestigious grants, such as the "special distinguished" grants (*tokubetsu suishin kenkyuhi*) of the Ministry of Education, Science, Sports and Culture (Monbusho).

For most of the ministry's common grants-in-aid of research (equivalent to NIH general research grants), there are only a few reviewers in each broad research field and they are asked to read and mark at least a few hundred applications without having any meeting among themselves. Many of the applications are outside the speciality of the reviewer. Partly as a result of that, the grant application form is generally short and brief. Only a very few special large types of grant are reviewed by committee meetings.

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This is obviously very different from Canada's MRC whose 40 study sections of 10 to 15 members each meet twice a year for 2–3 days to review some 50 applications each, or NIH's 100 study sections of 10–20 members each that meet three times a year to deal with 50 to 70 applications each time. I wonder what percentage of the grant budget is spent on these review processes in Canada and the United States?

The Japanese granting agencies undoubtedly spend far fewer resources on the evaluation process. No comments or suggestions are sent back to the applicants, so there is no way for them to know why their application has been successful or unsuccessful.

For most of the applicants, the review process is almost like a lottery, winning and losing without any reason other than luck. This is also quite different from the North American system where the applicants receive extensive comments from the study section.

Monopoly (or oligopoly) of the distribution of grants among the institutions discussed in the News article is not a unique problem of this new category of grant. Among the 68 large group grants for research in natural science selected as "priority research areas" (*juten kenkyuhi*) by Monbusho for 1996 and 1997, 37 and 32 per cent respectively of their 'leaders' are

affiliated with the University of Tokyo, 12 and 15 per cent respectively are from Kyoto University, while Osaka University, Nagoya University and Tohoku University account for 10 per cent each. Thus only 25 per cent is distributed among the other 93 national universities (not to mention municipal, prefectural and private universities).

Fundamental reform is required in these two areas of difference. Otherwise, Japan's rapidly increasing budgets for science will merely result in more money being poured into the already rich universities. And the increased funds will be useless for improving the foundations of Japanese science. It is not enough just to complain about the unfairness of one particular type of big grant.

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## Cultural credits

SIR — In her review of my book, *The Prehistory of Sex: Four Million Years of Human Sexual Culture* (*Nature* **383**, 683; 1996), Yvonne Marshall accuses me of poor referencing and of falsely claiming certain ideas as my own, such as my insis-

tence on the critical importance of the invention of the baby-carrying sling in human biocultural evolution. In fact, in 32 pages of endnotes and more than 440 references, I fully credit the work of those whom she claims I ignore (pages 273, 275, 276)<sup>1–4</sup>.

Marshall's frustration at not being able to pigeonhole me or my arguments may stem from the fact that they are not purely cultural as she implies, but biological too. Anyone with passing knowledge of primatology would know that the idea that penis size increased while clitoris size decreased in early hominid evolution is uncontroversial<sup>5,6</sup>, although the reasons for it are not<sup>7</sup>.

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"Purity, simplicity and time savings: a product that gives you all these, lets you take a break from the lab for a bagel, coffee and...hmm, maybe a haircut?" Kyungsun Suh, Ph.D., at the



# I think I know that face...

SIR — Exactly what do we recognize in a face? Intuition suggests that it is the eyes, nose and mouth — they, after all, are what the dictionary uses to define a 'face'. Portraitists labour to get these features right and poets describe the eyes as sure betrayers of identity. Computer scientists, not to be outdone, have designed vision systems that rely on precise measurements or templates of these 'internal' features to recognize faces<sup>1</sup>. This view often seems justified.

But some images, such as the one shown here, suggest that there might be more to face recognition than just an analysis of the internal facial features. The image

IMAGE  
UNAVAILABLE  
FOR COPYRIGHT  
REASONS

Dirck Halstead for Time

## Democrat coalition.

seems to be a fairly run-of-the-mill picture of the president of the United States, Bill Clinton, and the vice-president, Al Gore. A closer examination, however, reveals that the two are absolutely identical in terms of the eyes, nose and mouth and their spatial arrangement. (This was arranged by digital manipulation of the original photograph.)

Clearly, in this case, identification rests more on the appearance of the head as a whole than on the internal features alone. (Context is probably also of some significance in this case.) Manipulations with other images point to the same conclusion.

We therefore present this image in support of the idea that, at least on some occasions, the processing performed by the visual system to judge identity is better characterized as 'head recognition'<sup>2-4</sup>. Artificial

recognition systems might stand to benefit by incorporating, in some measure, the head processing strategy.

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## Chinese ethics

SIR — Since the Chinese Maternal and Infant Health Care Law, previously called the "eugenics and health protection law", was first proposed in December 1993, and finally promulgated on 27 October 1994, it has provoked widespread concern in the international scientific community (see *Nature* **367**, 1-3 & **372**, 123; 1994; **377**, 7 & **378**, 594; 1995; **383**, 204 & 569; 1996).

What is the attitude of Chinese geneticists towards ethical issues, including the controversial 'eugenics law'? A national survey, funded in part by the Ethical, Legal, and Social Implications Branch of the US National Center for Human Genome Research, was conducted among 402 geneticists from 30 provinces and autonomous regions in China.

The survey showed that 95 per cent of Chinese geneticists agreed that "people at high risk for serious disorders should not have children unless they use prenatal diagnosis and selective abortion"; 90 per cent agreed with the statement that "an important goal of genetic counselling is to reduce the number of deleterious genes in the population"; and 90 per cent called for ethical guidelines for genetics practice and research in China. Eighty-nine per cent supported current Chinese laws on abortion for genetic abnormalities and non-medical indications.

More than half opposed sex selection by any means; 55 per cent thought that gene donors should have a right to share in the profits from commercialized tests or treatments developed from using their genes; and 50 per cent thought that public education on genetics should be the top priority of the Chinese government health budget.

This is the first survey to provide a basis for international discussion on ethics and genetics in China.

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## Power games in Austrian science

SIR — The recent debate in *Nature* about Austrian biomedical research has highlighted several shortcomings but has failed to identify the cause of the problem. I think Austrian science suffers mostly from organizational defects in the university system.

The faculties are run by an oligarchy of committee members who are elected irrespective of their research record. Academic decisions are taken by committees whose voting members consist of 50 per cent professors, 25 per cent academic staff and 25 per cent students. Those eligible for committee work include all professors and a number of student representatives and academic staff delegates who are often re-elected for a limitless number of terms. Scientific competence is not a criterion in the election process and hence of low priority in the committee work.

Scientific qualification is evaluated by committee members who lack research competence. Candidates for academic promotion, tenure and professorships are evaluated and selected by delegates who may have no or only a limited record of research performance. The same applies to the allocation of faculty resources, which may be decided by delegates who have never ventured into drafting a research proposal or beyond.

The committee groups favour their members for academic promotion. In the faculty with which I am affiliated, most professorships in the past decade have been awarded to members of the committee oligarchy, with promotion of other candidates from within the faculty or outside the university being rare exceptions. As a result, indifference to science is propagated from the lower to the higher ranks of the faculty.

The committee oligarchy is liable to misuse university resources. Decisions are all too often traded between the committee groups to secure their interests, but are not taken according to objective criteria in the interest of the university.

University funds are distributed irrespective of research performance, because performance in general is not evaluated.

Correction of these shortcomings should be a priority for the Austrian Ministry of Science in its struggle to make our universities fit to compete for European Union research funds. That Austrian science still survives is largely due to the Fonds zur Förderung der Wissenschaftlichen Forschung, the national agency for research funding, which keeps to internationally accepted principles of peer evaluation.

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# Reversal of fortune for nascent proteins

Juan S. Bonifacino

**The demonstration of a way in which cytomegalovirus can evade immune surveillance also points to a mechanism by which abnormal proteins, once synthesized, can be degraded.**

LARGE DNA viruses such as adenovirus, herpes simplex viruses 1 and 2, Epstein-Barr virus and cytomegalovirus have evolved sophisticated mechanisms to avoid detection by their hosts' immune systems<sup>1</sup>. Most of these mechanisms are geared towards preventing the expression of antigenic viral peptides that are bound to major histocompatibility complex (MHC) class I molecules, on the surface of the infected cells (antigen presentation). In this way, the viruses turn off the signal that would alert the immune system to the presence of an intracellular pathogen and, undetected by antigen-specific T lymphocytes, the infected cells continue to provide a safe haven for the viruses.

The article by Wiertz *et al.* on page 432 of this issue<sup>2</sup> describes a new addition to the growing list of viral gene products that interfere with antigen presentation by MHC class I molecules (see table). The human cytomegalovirus *US2* gene product, like the previously described *US11* protein<sup>3</sup>, accomplishes this feat by transporting (or dislocating) newly synthesized MHC class I molecules from the endoplasmic reticulum into the cytosol. Strikingly, dislocation involves retrograde transport through the translocon, which is the protein-conducting channel responsible for insertion of polypeptide chains into the endoplasmic reticulum as they are being synthesized. When they are released into the cytosol, the dislocated proteins are quickly destroyed by the proteasome, which is a multi-subunit proteolytic complex. Wiertz *et al.* also show that misfolded MHC class I molecules undergo a similar fate, even in the absence of the *US2* protein. So these findings not only shed light on a pathologically relevant aspect of virus-host interactions, but they also point

to the mechanisms by which cells eliminate abnormal newly synthesized proteins from the endoplasmic reticulum.

Proteins that are destined for secretion from the cell or for localization to

proteins undergo a series of modifications, including glycosylation, folding, disulphide-bond formation and oligomeric assembly. Failure to fold properly or to assemble into oligomers generally results in impaired

transport from the endoplasmic reticulum to the Golgi complex and to other organelles of the secretory pathway (Fig. 1). Often, the proteins that are retained in the endoplasmic reticulum are degraded, and this ability to retain and degrade abnormal proteins has been referred to as the 'quality control' function of the endoplasmic reticulum<sup>5</sup>.

The notion that the retained proteins can be rapidly degraded has its origin in the work of Sidman<sup>6</sup>, who showed that mutant immunoglobulin (IgM) molecules produced in B-cell hybridomas are quickly destroyed intracellularly. Many studies over the past decade have established that these initial observations apply to a variety of misfolded, unassembled

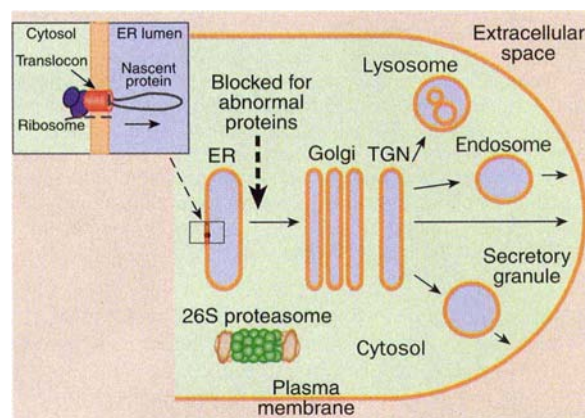


FIG. 1 Intracellular routes followed by proteins of the secretory pathway. Newly synthesized proteins enter the secretory pathway at the endoplasmic reticulum (ER) by translocation through the translocon (inset). After post-translational modification in the lumen of the endoplasmic reticulum, they move to the Golgi complex and the trans-Golgi network (TGN). From here, proteins are transported to lysosomes, endosomes, secretory granules, the plasma membrane or the extracellular space. Abnormal proteins cannot usually reach the Golgi complex and they are often degraded.

organelles of the secretory pathway begin their journey when, at the same time as they are synthesized, they are transported from the cytosol into the lumen of the endoplasmic reticulum (Fig. 1). Known as translocation, this process is mediated by the translocon, which is a cylindrical structure with a central pore, composed of 3–4 copies of the Sec61 heterotrimeric complex<sup>4</sup>. Once they reach the lumen of the endoplasmic reticulum, the nascent

or aberrantly modified proteins, which are also degraded soon after synthesis<sup>7</sup>. Interest in this phenomenon grew with the finding that many human hereditary diseases, such as  $\alpha_1$ -antitrypsin deficiency and cystic fibrosis, arise from the retention and degradation of mutant gene products in the endoplasmic reticulum. Even normal proteins such as apolipoprotein B, T-cell antigen receptor chains and MHC class I molecules succumb to this type of degradation under certain metabolic, developmental or pathological conditions<sup>6</sup>. But the transport of these proteins to lysosomes — a major site of intracellular protein breakdown — is not required for degradation, and the degradative process is insensitive to inhibitors of lysosomal hydrolases.

If lysosomes are out of the picture, where are the proteins that have been retained in the endoplasmic reticulum degraded? Attempts to identify hydrolytic activities capable of complete protein breakdown within the lumen of the endoplasmic reticulum were largely fruitless. So researchers set their sights on the cytosol,

**Viral proteins that interfere with antigen presentation by MHC class I molecules**

| Virus                        | Viral protein | Primary effect                                                                              |
|------------------------------|---------------|---------------------------------------------------------------------------------------------|
| Adenovirus                   | E3-19K        | Retains MHC class I molecules in the ER                                                     |
| Herpes simplex virus 1 and 2 | ICP47         | Blocks transport of antigenic peptides from the cytosol into the endoplasmic reticulum (ER) |
| Epstein-Barr virus           | EBNA4         | May block generation of antigenic peptides or their transport from the cytosol into the ER  |
| Cytomegalovirus              | US3           | Retains MHC class I molecules in the ER                                                     |
| Cytomegalovirus              | US11          | Dislocates MHC class I molecules from the ER into the cytosol                               |
| Cytomegalovirus              | US2           | Dislocates MHC class I molecules from the ER into the cytosol                               |

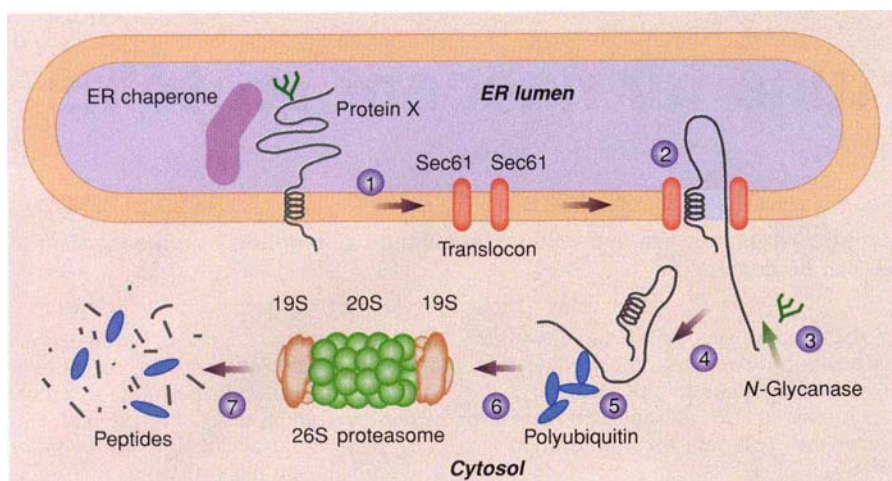


FIG. 2 Degradation of a generic protein (protein X) retained in the endoplasmic reticulum (ER). (1) A putative chaperone forces the association of protein X with the translocon; (2) reverse translocation from the lumen of the endoplasmic reticulum to the cytosol through the translocon; (3) deglycosylation by a cytosolic *N*-glycanase; (4) release of the protein into the cytosol; (5) polyubiquitination; (6) targeting to the proteasome; (7) degradation. The scheme is based on data from Wiertz *et al.*<sup>2</sup> and several other publications<sup>3,8–13</sup>, and not all of the steps have been demonstrated for all substrates of this pathway. A membrane-bound protein is used as an example but soluble, luminal proteins probably follow a similar pathway. Membrane-bound US2 cytomegalovirus protein would behave like the chaperone shown in the scheme, except that it would escort MHC class I molecules throughout the entire pathway. Several of the steps, including 2, 5, 6 and 7, require ATP.

where another major degradative pathway, the ubiquitin–proteasome pathway, exists. Several groups have shown that various proteins that have been retained in the endoplasmic reticulum are indeed degraded by the proteasome<sup>3,8–12</sup>, in some cases following polyubiquitination<sup>9,10,12</sup>. For this process to occur, proteins must be transported back into the cytosol: this has been demonstrated for both soluble<sup>13</sup> and membrane-bound proteins<sup>3</sup>. And now Wiertz *et al.*<sup>2</sup> show that MHC class I molecules bind to — and are likely to be translocated back to the cytosol by — the Sec61-containing translocon.

Figure 2 depicts a possible sequence of events during the degradation of a generic protein (protein X) that has been retained in the endoplasmic reticulum. Proteins that are targeted for destruction are taken back to the translocon, either by chaperones within the endoplasmic reticulum or, in the case of viral infection, by virally encoded proteins (step 1). The translocon then provides the conduit through which

proteins are extruded back into the cytosol, in a process known as reverse translocation (step 2). Once exposed to the cytosolic milieu, proteins are quickly deglycosylated by an *N*-glycanase (step 3), released from the endoplasmic reticulum membrane (step 4) and polyubiquitinated (step 5). They are then targeted to the 26S proteasome (step 6),

#### INTERSTELLAR MEDIUM

## Do two photons make a band?

Theodore P. Snow

THE delicious conundrum offered us by the unexplained diffuse interstellar bands (DIBs) has deepened with a recent announcement from Peter Sorokin and James Glowina (to be published in the *Astrophysical Journal* on 20 December). Sorokin and Glowina, experts in laser physics and molecular spectra, have ventured into a long-standing astronomical mystery: searching for the source of some 200 interstellar absorption features, the first of which was recognized<sup>1</sup> as long ago as 1921.

These features, seen in the form of weak, sometimes broad, absorption troughs against the continuous spectra of hot background stars, clearly have a lot to tell us about the physics and chemistry of the diffuse interstellar medium. But extracting the information that is being offered will mean identifying the carrier particles, and there's the rub.

Molecules can superimpose a band structure on each electronic transition

which hydrolyses them to small peptides (step 7). The details of this process might vary for different proteins. For instance, the US2 protein of cytomegalovirus accompanies MHC class I molecules to the cytosol and is itself degraded by this pathway<sup>2</sup>. And whereas polyubiquitination precedes the degradation of some proteins, this has not yet been shown to be the case for MHC class I molecules<sup>2</sup>.

Many questions remain unanswered and will surely be the subject of future research. How is the translocon reassembled and reopened for reverse translocation? What provides the driving force for retrograde transport through the translocon? Are the reverse translocation and the degradation steps spatially or functionally coupled? Are cytosolic chaperones involved in this process? And is ubiquitination an obligatory requirement for degradation? Whatever the answers, it has become clear that the once poorly understood phenomenon of protein degradation from the endoplasmic reticulum uses well-known components of the transport and degradation machineries. Moreover, while we are just beginning to understand this process, cytomegalovirus must have known about it all along — or at least since it evolved the ability to evade the immune system. □

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because of rotational and vibrational transitions, and in recent years it has begun to appear that complex organic molecules would prove to be the culprits. The detection of possible rotational and vibrational band structure<sup>2–4</sup>, the widely accepted evidence<sup>5–7</sup> for both carbon-chain and aromatic molecules in the interstellar medium, laboratory spectra of analogous species embedded in solid matrices<sup>8–10</sup>, and inconsistencies with proposed solid-state processes<sup>11</sup> have all helped to lead us down that path. But now Sorokin and Glowina have considered something that has long been ruled out in most minds as a potential DIB carrier: molecular hydrogen.

They envisage a cloud close to a hot star in which an intense field of ultraviolet photons, created by a combination of resonance scattering and Raman scattering, maintains a population of excited H<sub>2</sub> molecules that produces the DIBs by absorbing visible-wavelength photons. As support for this complex scheme, they



calculate the wavelengths at which the excited hydrogen molecules can absorb, and find close matches with a number of observed DIBs.

The model invokes a two-photon process that can only occur in regions of very intense UV-radiation fields.  $H_2$  molecules must reach excited electronic states, from which they can produce optical DIB absorption as long as the requisite visible-wavelength photon arrives before the molecule relaxes to its ground state (which takes only nanoseconds).

Sorokin and Glownia argue that sheet-like clouds near hot stars could trap UV flux internally through resonance scattering in the strong electronic absorption bands, so that visible-wavelength photons would have a reasonable chance of encountering excited  $H_2$  molecules. The few very broad DIBs would result when the second absorption also destroyed the molecule by providing enough energy to break it apart.

In cases where the model fails to produce enough UV flux, they add another ingredient: Raman scattering, a process that can convert far-UV photons into two or more lower-energy photons. In the most complex part of their theory, the authors claim that UV photons at 91.7 nm Raman-scatter from  $H_2$  molecules to produce three secondary photons, including one at 110.9 nm which then stimulates the excited state in  $H_2$  from which the 579.7-nm DIB is produced.

This picture is very tempting; it involves real physical processes that can in principle be reproduced in the laboratory, and it has the great attraction that it uses the most abundant interstellar molecule of them all. But unfortunately, several serious theoretical and observational inconsistencies militate against the model.

First, it is very hard to believe in an interstellar UV field intense enough to allow the proposed two-photon process to occur. Even close to a hot star, and allowing for the UV-trapping mechanism (which is a generous allowance), it seems impossible to create enough of an excited population to account for the DIB absorption<sup>12</sup>. And one would expect these trapped UV fields to light up the clouds near hot stars with a diffuse UV glow (peaking at the wavelengths of the strong resonance bands of  $H_2$ ), but nothing like that has been reported.

From the observational side, perhaps the most serious objection is that the real DIBs do not correlate with the kinds of environments required in the Sorokin and Glownia model; on the contrary, the DIBs quite happily appear in the spectra of stars that are far too cool to produce the necessary UV flux. Worse, they show an abhorrence<sup>13</sup> for the very regions (nebulousity close to hot stars) where this model would have them formed most efficiently.

The model also requires substantial equilibrium populations of excited  $H_2$ . But despite intensive searches, only extremely small quantities have been seen<sup>12</sup>, and there is no evidence for the predicted UV emission features, including the one at 110.9 nm (in a spectral region well observed by the Copernicus satellite in the mid-1970s).

Even the spectroscopic underpinning is weak: many of the wavelength coincidences Sorokin and Glownia use to support their model lie outside the observational uncertainties and are therefore not coincidences at all. A recent laboratory study<sup>14</sup> of the proposed two-photon transitions in  $H_2$  found some encouraging results, but they are not entirely consistent with Sorokin and Glownia's hypothesis.  $H_2$  was indeed found to produce two-photon absorption features in the optical regime, and some bands were observed to closely match known DIBs, but an equal number were found to miss the DIB wavelengths by more than the experimental or observational uncertainties. Given the large number of DIBs and the immense complexity of the  $H_2$  spectra, some matches will occur by coincidence.

So where does this leave us? The new mechanism, in its current form, seems not to be viable within the astronomical constraints. Yet it is tantalizing, and one wants to encourage the kind of creative thinking that went into it, for perhaps the

obstacles will be surmounted and such a model will eventually be vindicated. On the other hand, the complexity of this model, along with the inherent difficulty of sorting out exactly which organic molecules might be the DIB carriers under the more widely accepted view, persuades us that we still have a long way to go in solving this mystery. □

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## HUMAN GENETICS

# Transcribing diabetes

John A. Todd

ALMOST every person reading this article will know of somebody who suffers from diabetes — at a frequency of about 3 people in 100 it is one of the world's most common inherited diseases. Unfortunately, inheritance of the common forms of diabetes is not simple, making it difficult to identify the genes responsible for the disease<sup>1,2</sup>. However, in about 1–2% of cases, diabetes is inherited in a classic autosomal dominant pattern: anyone in a family who has one copy of the defective gene develops hyperglycaemia (increased levels of blood glucose). This form of diabetes is known as maturity-onset diabetes of the young (MODY), and it usually develops before the age of 25 (ref. 3). The identification by Bell and colleagues<sup>4,5</sup> on pages 455 and 458 of this issue of not one, but two, *MODY* genes is news in itself. The fact that they are related transcription factors is unexpected, and adds an exciting new dimension to our knowledge of the pathogenesis of diabetes and to the hunt for genes that predispose to the more common forms of the disease (see table).

Diabetes is caused by the impaired pro-

duction of insulin by the pancreatic-islet  $\beta$ -cells and/or by a reduced response of tissues to insulin action (called insulin resistance). Pancreatic  $\beta$ -cells are the only cells in the body that secrete insulin, the hormone that ensures that blood glucose levels stay constant, below 140 mg dl<sup>-1</sup>. If  $\beta$ -cells are damaged or defective, hyperglycaemia ensues. Chronic hyperglycaemia damages tissues by an unknown mechanism, and it is a major cause of blindness, gangrene and kidney failure. The causes of  $\beta$ -cell failure or insulin resistance are largely unknown, but the identification of the genes responsible has, and will, provide insights into basic disease mechanisms and aetiology.

Bell was the first person to map a *MODY* gene to a chromosome by matching the transmission of the disease in a huge pedigree (the R-W family) to chromosomal markers spread throughout the 4,000 million base pairs of the human genome<sup>6</sup>. Known as genome scanning, this procedure has also been applied to common forms of diabetes<sup>1,2</sup>. *MODY1* was mapped to a 13-million-base-pair region



Different types of diabetes and the genes and mutations that cause or predispose to them

| Type of diabetes                                                | Gene/molecule                                                  | Function                                                                 | Effect of mutation                                                            | * Number of cases per 10,000 diabetics                                 |
|-----------------------------------------------------------------|----------------------------------------------------------------|--------------------------------------------------------------------------|-------------------------------------------------------------------------------|------------------------------------------------------------------------|
| MODY1                                                           | <i>TCF14</i> / HNF-4 $\alpha$                                  | Hormone receptor; regulates HNF-1 $\alpha$ expression                    | Reduces insulin response of $\beta$ -cell to glucose                          | <1                                                                     |
| MODY2                                                           | <i>GCK</i> / Glucokinase                                       | $\beta$ -cell glucose sensor                                             | " "                                                                           | 15                                                                     |
| MODY3                                                           | <i>TCF1</i> / HNF-1 $\alpha$                                   | Transcription factor expressed in liver and $\beta$ -cells               | " "                                                                           | 115                                                                    |
| Diabetes and deafness                                           | <i>mt tRNA<sup>Leu</sup></i> base 3243 / Transfer RNA molecule | Protein synthesis in mitochondria                                        | Reduces insulin production                                                    | <100                                                                   |
| Insulin resistance                                              | Insulin receptor                                               | Binds insulin and transduces insulin signal into cell                    | Insulin resistance                                                            | <10                                                                    |
| † IDDM1, type 1 (autoimmune, usually diagnosed under age 18 yr) | <i>HLA-DQB1</i><br><i>HLA-DRB1</i><br><i>HLA-DQA1</i>          | $\alpha$ - and $\beta$ -chains of MHC class II immune-response receptors | Autoimmune, T-cell destruction of $\beta$ -cells, absolute insulin dependency | 500 (Polygenic factor)                                                 |
| † IDDM2                                                         | Insulin VNTR                                                   | Promoter polymorphism at -596 of insulin gene                            | Affects insulin transcription                                                 | Unknown, but might regulate tolerance to preproinsulin, an autoantigen |

\* Type 2 diabetes accounts for 9,260 per 10,000 cases of diabetes, however, most of the affected genes/molecules have not been identified. Two type 2 diabetes genes that have been mapped are *NIDDM1* (ref. 2) and *NIDDM2* (ref. 14). *NIDDM2* is on chromosome 12q and could be *MODY3* (ref. 4). The relative frequencies of the types of diabetes are approximate and vary between different populations.

† IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus.

of chromosome 20q11.2–q13.1, containing perhaps about 250 genes. But *MODY1* is extremely rare<sup>7</sup>, and there were not enough family members with the condition to fine-map the *MODY1* gene to an interval that was small enough (about 3 million base pairs or 60 genes) to warrant sequencing genes from patients and healthy individuals. So it was there that the *MODY1* story ended — until now.

Clinical studies have shown that *MODY* is a defect in insulin secretion rather than in insulin action<sup>8</sup>. Good candidates for *MODY* genes are therefore those that are known to function in  $\beta$ -cell insulin production and secretion. Hundreds of genes are involved in these pathways, but the glucokinase gene (which encodes the glucose sensor of the  $\beta$ -cell) turned out to be *MODY2* (chromosome 7p13)<sup>9</sup>. Bell, Froguel and colleagues showed that mutations that inactivated the glucokinase gene caused the  $\beta$ -cell to secrete less insulin in response to glucose, leading to hyperglycaemia.

It was now clear that there was more than one cause of *MODY*, each one resulting from a different defective gene. Further genome scanning of *MODY* families led Froguel and colleagues to chromosome 12q24 and *MODY3* (ref. 10). *MODY3* is much more common than *MODY1* (see table), and enough families became available through international collaborations to map the gene to about 3 million base pairs<sup>4</sup>. In the new work, Bell and colleagues cloned this region, and began to characterize the genes contained within it<sup>4</sup>. After sequencing only ten of the 60 genes that could be in the interval, they hit the jackpot with the gene that encodes hepatocyte nuclear factor-1 $\alpha$ , HNF-1 $\alpha$ . HNF-1 $\alpha$  is a transcription factor that is involved in gene expression in the liver<sup>11</sup>, but it is also expressed in pan-

creatic  $\beta$ -cells and has been shown to activate transcription of the rat insulin gene<sup>12</sup>. They found seven different HNF-1 $\alpha$  mutations in seven unrelated *MODY3* families.

It must have been rather breathtaking for the Bell lab when they realized that a positive regulator of HNF-1 $\alpha$  expression, the steroid-hormone-receptor HNF-4 $\alpha$  (refs 11, 13) was encoded right on top of their old adversary *MODY1*. When they sequenced the *HNF-4 $\alpha$*  gene from members of the R-W *MODY1* pedigree, they found a nonsense mutation that predicted a 267-amino-acid truncated protein lacking the regions involved in dimerization and transcriptional activation<sup>5</sup>. One lesson from this work is that even if the interval is large, known genes in the interval should be sequenced, and genes lacking a strong case for being functional candidates should not be neglected.

What next? The biological effects of mutant forms of HNF-1 $\alpha$  and HNF-4 $\alpha$  are unknown, and finding out what they do in  $\beta$ -cells is now a key research target. Homozygous knockouts of *HNF-1 $\alpha$*  and *HNF-4 $\alpha$*  are lethal in mice, and largely uninformative<sup>4,5</sup>, but analysis of  $\beta$ -cell function, insulin secretion and  $\beta$ -cell mass<sup>8</sup> in heterozygous mice should be instructive. Another question is whether common, less damaging mutations of *HNF-1 $\alpha$*  and *HNF-4 $\alpha$*  (and of other family members) predispose to common forms of diabetes, late-onset type-2 diabetes<sup>2,14</sup> and autoimmune, insulin-dependent type-1 diabetes<sup>1</sup>. And which genes modify the severity of *MODY*; for example, mutations that increase the sensitivity of tissues to insulin? Up to 20% of *MODY* families remain unexplained — what other unexpected genes will be found? HNF-4 $\alpha$  is an orphan receptor, that is, we do not know what its ligand is<sup>13</sup>. Only

recently was the ligand for the PPAR $\alpha$  hormone superfamily member identified as leukotriene B<sub>4</sub>, intriguingly linking lipid metabolism with inflammation<sup>15</sup>. Will identification of the ligand for HNF-4 $\alpha$  produce a similarly startling link, and do its levels and activity influence susceptibility to diabetes?

Because *MODY2* is relatively mild outside pregnancy, there has been virtually no clinical impact following the discovery that mutations of glucokinase cause the disease. But this finding has prompted the pharmaceutical industry to invest in new diabetes drug-discovery programmes. Mutations in *MODY3* produce a more pronounced hyperglycaemia than those in *MODY2*, and this can lead to the typical complications of blindness, gangrene and kidney failure. Moreover, it is thought that hyperglycaemia itself produces irreversible changes in  $\beta$ -cell function, implying that early detection, and treatment by diet and exercise, may well be beneficial. □

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# How Earth created heaven

James H. Knapp

THAT the Earth's surface consists of rigid plates that travel the globe and interact along their boundaries was a central and highly successful tenet of plate tectonic theory in the 1960s. But whereas the oceanic plates fit this model well, their continental counterparts, with their thicker, lighter and weaker crust and upper mantle (together defining the lithosphere), appear to deform at large distances from active plate boundaries. The knowledge that continents deform internally is not particularly new. But on page 450 of this issue<sup>1</sup> Abdrakmatov *et al.* report surprisingly high modern-day shortening rates across the Tien Shan, or 'Heavenly Mountains', of Central Asia, far from the active collision zone between India and Eurasia.

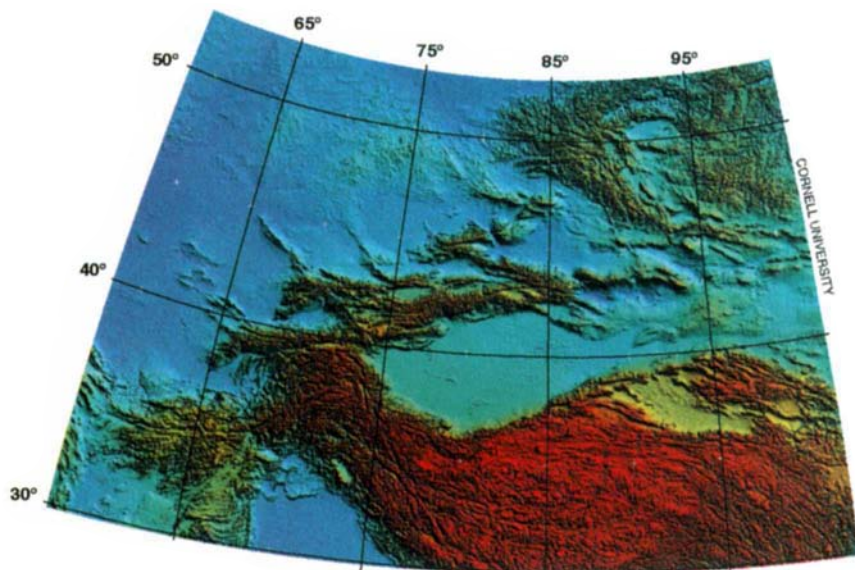
The satellite-based Global Positioning System has high resolution, and it allows measurement of crustal-scale deformation on a timescale of a few years. Using it, Abdrakmatov *et al.* found shortening rates of  $\sim 20 \text{ mm yr}^{-1}$  across the central Tien Shan, in the Central Asian republics of Kyrgyzstan and Kazakhstan. Although these rates are moderate compared with measurements from other areas, such as the South Pacific<sup>2</sup>, they still account for a large percentage of the total convergence of  $\sim 45 \text{ mm yr}^{-1}$  between India and Siberia<sup>3</sup>, implying that the forces of colliding continents are being transferred far from what was once the Eurasian plate margin.

Moreover, the coincidence of the measured shortening direction with the relative motion of the Indian and Eurasian plates<sup>3</sup> suggests that these forces are transmitted in a geometrically straightforward way, despite the striking heterogeneity of the crust in this area. In fact, the growth of the Tien Shan within a much larger region composed of a complex assemblage of different geological units<sup>4</sup> adds to the enigma. It may be that old scars in the crust from previous episodes of tectonic upheaval make the region relatively weak (in the figure, note the large faults extending north-westward from the range). But only some faults are reactivated, raising the question of how and why the strain is localized where it is. The answer may in part lie in features such as the Tarim Basin (the low area between the Tien Shan and the Tibetan plateau), which could be a preserved block of denser, stronger oceanic crust, caught in the collisional collage.

How the horizontal crustal movements get translated into the lofty peaks of the Tien Shan hinges largely on the steepness of the faults and the depths to which they descend in the crust — factors that are largely unknown in this region. What is apparent is that deformation does not propagate in a regular and predictable

fashion away from the plate boundary.

The shortening rates are significantly greater than estimates derived from analysis of either the surface geology<sup>5</sup> or earthquake slip on faults<sup>6</sup>. Such a discrepancy could indicate that the faults in these mountains are creeping instead of causing earthquakes; equally, it could mean that large stresses are building up in the region, posing a considerable seismic threat.



Topographic image of the Tien Shan (centre) and Tibetan plateau (bottom). The Tien Shan is far from the former southern margin of Eurasia (the southern edge of Tibetan plateau), and isolated within the continental interior. Red marks high elevations, blue low.

If these modern shortening rates are representative of the geological past, one is left with the impression that the Tien Shan have virtually leapt skyward. By drawing on geological estimates for the total amount of shortening, and assuming that the present is the key to the past, Abdrakmatov *et al.* speculate that the deformation may have begun less than 10 million years ago.

A word of caution is in order, however, because the extrapolation of crustal deformation rates from decades to geological timescales ( $10^4$ – $10^7$  yr) remains questionable<sup>7</sup>. In the case of the Tien Shan, deposition of thick sections of sediment in adjacent areas<sup>8</sup> suggests that the mountains were rising many tens of millions of years ago, even before the collision of India with Eurasia. Similarly, thermal histories of rocks now at the surface show that they were rising, cooling and being eroded some 20 to 30 million years ago, implying that the Tien Shan probably has a history at least that long<sup>9</sup>.

Even though the topographical evidence of such an earlier phase of mountain building may have been eroded away before the latest uplift, estimates of the total amount of shortening probably include part of that

earlier history. These new data may imply that the deformation and ascent of the Tien Shan has accelerated markedly in the past few million years.

On a larger and perhaps more fundamental scale, it is unclear how this crustal shortening is accommodated in the underlying mantle lithosphere. Seismological evidence from the central Tien Shan<sup>10</sup> implies that rather than being a thickened lithosphere floating on a cold, stiff mantle, these mountains may be supported by a relatively hot, weak mantle plume rising from below. If such thermal weakening is a long-lived feature beneath the Tien Shan, it

may have played a key role in focusing the effects of continental collision here<sup>11</sup>.

As one of the youngest and highest mountain belts in the world, the Tien Shan might be viewed as the tip of a continental iceberg; rising above the Central Asian steppe, they belie an intriguing mystery at depth, whose solution would tell us much about the mechanical nature and evolution of the continents we inhabit. □

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# Flies go with the flow

Mandyam V. Srinivasan

ANYONE who has watched a fly perform a flawless landing on the rim of a teacup will know that even relatively small and 'simple' nervous systems can orchestrate impressive visuomotor coordination. To accomplish such manoeuvres — or even merely to move in a straight line — a flying creature needs to be able to keep track of its own motion by monitoring the apparent motion of its surroundings as it moves. Understanding exactly how this is achieved has challenged neurobiologists for decades. The paper by Krapp and Hengstenberg<sup>1</sup> in this issue (page 463) sheds new light on how the pattern of image motion that is experienced by the moving eye is analysed by the nervous system of the fly (*Calliphora erythrocephala* Meig.) to deduce motion in three-dimensional space and at a given instant in time.

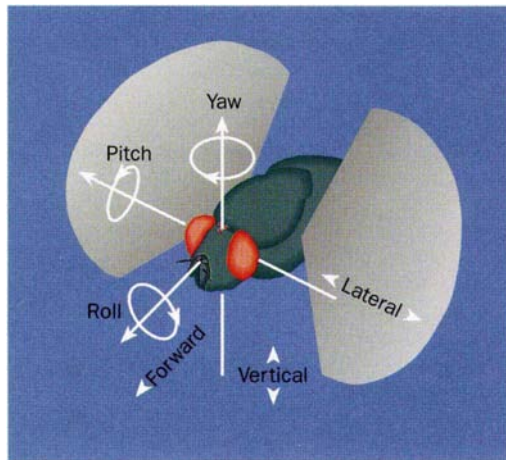
A flying insect can move with six degrees of freedom: it can yaw, pitch or roll, as well as translating forwards, laterally or vertically (see figure). In principle, these motions can be inferred from the patterns of image motion, or 'flow fields', that they create in the eyes. For example, yaw generates forward motion of the image in one eye, backward motion in the other, and no motion either directly overhead or directly below. Forward translation produces backward motion of the image in both eyes, and no motion in the frontal or rearward regions of the visual field. Roll causes upward motion of the image in one eye and downward motion in the other, and, again, there is no motion in the front or rear.

These patterns of image motion are thought to be detected by a handful of neurons in a region of the fly's brain known as the lobula plate<sup>2</sup>. These neurons have large visual fields and they are highly sensitive to motion stimuli from the eyes. Krapp and Hengstenberg have used a new technique to map the receptive fields and response properties of these neurons in a very precise fashion. Their results indicate that each neuron is exquisitely 'tuned' to sense the pattern of image flow that results from a specific type of motion of the animal.

The key feature of the new technique is the visual stimulus, which allows the experimenter to determine — precisely and rapidly — the flow field to which each neuron is tuned. The stimulus consists of a spot that moves at a constant speed in a small, circular trajectory. Typically, a neuron in the lobula plate shows a fluctuating response to such a stimulus.

The maximum sensitivity of the neu-

ron to image motion (within the small region of the receptive field that is stimulated by the rotating spot) is determined by the difference between the largest and the smallest values of the response, measured over one cycle of rotation of the spot. The 'preferred' direction of image motion within this region is determined by the direction in which the spot is moving when the response is maximal



Flight control — a flying insect can move within six degrees of freedom and, in the paper discussed here, Krapp and Hengstenberg<sup>1</sup> analyse the way these movements are interpreted in terms of patterns of image motion by a fly's nervous system.

(after appropriate correction for the delay in the response). So the local sensitivity of the neuron to image motion can be characterized by a vector whose magnitude represents sensitivity, and whose direction represents the locally preferred direction. These measurements can be repeated at different regions in the visual field by repositioning the stimulus, which is carried on a calibrated cardan arm. The final result is a group of vectors that describes the pattern of image motion to which the neuron is tuned.

Krapp and Hengstenberg<sup>1</sup> have used this method to investigate one class of neurons in the lobula plate, the so-called VS neurons. Until now these neurons were thought to respond simply to image motion in the vertical direction<sup>2</sup>. The new results show that these neurons are more specifically tuned — they respond most strongly to rotation about an axis in the horizontal plane. One subset of the VS neurons responds best to rotations about the fly's long axis (roll); another subset is stimulated by rotations about a transverse axis (pitch); and yet another responds best to rotations about an axis lying between these two. Taken together, the responses of these neurons can encode rotations

about any axis in the horizontal plane.

Another neuron, Hx, is maximally silenced when the fly moves in the forward direction. Together with neurons that are tuned to other directions of translation, Hx could, in principle, encode the direction of translation in three dimensions. But further work is needed to discover and characterize all of the neurons that might be involved in evaluating self-motion.

These findings raise important questions as to the number and types of neurons that are involved in computing self-motion; precisely how information on self-motion can be encoded by the pattern of activities in such an ensemble of neurons; the minimum number of neuron types that would be necessary for this purpose; and the role of any redundancy. It would also be interesting to compare results in flying insects — many of which move with six degrees of freedom — with results in walking insects (such as ants), which are restricted to forward translation and yaw. Finally, many of these considerations could be valuable in the design of vision-based algorithms for the navigation of mobile robots.

The discovery that motion-sensitive neurons in the lobula plate of the fly are tuned to respond to specific flow fields is echoed by similar findings in vertebrates. For example, there are neurons in the nucleus rotundus of the pigeon brain<sup>3</sup> and in the medial superior temporal area of the monkey brain<sup>4</sup>, that signal an approaching object by responding specifically to expanding images. The emerging principle, which was clearly foreshadowed about a decade ago<sup>5</sup>, seems to be that the brain makes sense of the chaotic world by viewing it through sensory filters that are 'matched' to meaningful external events. □

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# Programmed to stay together

Sharyn A. Endow

THE fungus gnat *Sciara coprophila* is a dipteran (two-winged) insect like the fruit fly *Drosophila*. But unlike *Drosophila*, *Sciara* undergoes programmed chromosome loss as part of its normal mechanism of sex determination during early development. Although the unusual behaviour of *Sciara* chromosomes during embryogenesis has received little attention over the past 20 years, de Saint Phalle and Sullivan<sup>1</sup> have now elegantly extended previous observations in a study published in *Development*. Using laser-scanning confocal microscopy and fluorescence *in situ* hybridization, they have looked at chromosome elimination during early *Sciara* development. And their results can be understood in the context of current ideas about chromosome segregation in more 'conventional' organisms.

Classical studies by Metz (reviewed in refs 2, 3) first showed that the germ cells of male and female *S. coprophila* contain the same number of chromosomes, but the somatic cells have fewer chromosomes. These differences are due to losses that occur in the *Sciara* early embryo, and they are the basis of its unusual sex determination (Fig. 1). Zygotes contain three X chromosomes: one from the oocyte and two from the fertilizing sperm. The sperm contributes two X chromosomes because of a failure to separate (known as nondisjunction) during spermatogenesis<sup>2,3</sup>. The loss of one X chromosome from diploid nuclei in early embryos results in an X/X female; and the loss of two X chromosomes gives rise to an X/0 male. This programmed chromosome loss also causes the elimination of the so-called L (limited) chromosomes.

Early studies by Du Bois<sup>4</sup> showed that chromosome loss occurs by arrest of the eliminated chromosomes on the mitotic spindle. Because the X and L chromo-

somes are lost after the zygote has undergone several divisions, each of the diploid nuclei must lose the same number of chromosomes for the somatic cells to be of the same sex and to have the same chromosomal constitution.

Using confocal microscopy and a method of fluorescence *in situ* hybridization that had been developed for *Drosophila*<sup>5</sup>, de Saint Phalle and Sullivan followed the X chromosome during early cleavage-divisions of *Sciara*. They used an X-chromosome-specific molecular probe that allowed them to see that the first abnormality shown by the eliminated X chromosomes is the failure of sister chromatids to separate completely (Fig. 2). The chromosomes remain joined at a region on the long arm of the X chromosome, rather than at the centromere or the telomere.

Surprisingly, the centromeres of the eliminated X chromosomes remain attached to the spindle during anaphase — the sister chromatids would normally be expected to separate and migrate towards opposite poles of the spindle. But despite their arrest on the metaphase plate, the chromatids seem to be under tension, as though they are nonetheless subjected to the forces that cause the remaining chromosomes to move polewards. This is supported by the fact that, occasionally, when one centromere becomes detached from the spindle, both chromatids migrate to a single pole. So the X chromosome cannot move from the metaphase plate because the sister chromatids do not separate completely. The authors also observed the loss of the L chromosomes in divisions preceding loss of the X chromosome, and they found that the L chromosomes are eliminated in the same way, by a failure of the sister chromatids to separate.

How are specific chromosomes programmed to undergo nondisjunction and loss? Elimination of the X chromosomes during the early embryonic divisions of *Sciara* is controlled by maternal factors, and previous studies by Crouse<sup>6</sup> showed that the process requires a *cis*-acting region of the X chromosome, known as the controlling element. This lies adjacent to the centromere of the X chromosome — a site that does not correspond to the region of failed sister-chromatid separation. Crouse also showed that the trans-

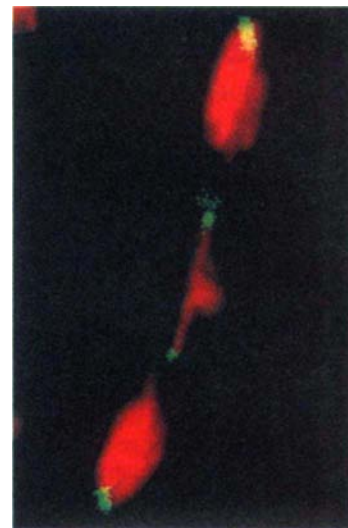


FIG. 2 Elimination of an X chromosome in a female embryo of *Sciara*. The bright dots of fluorescence mark the X chromosomes, identified using fluorescence *in situ* hybridization and a probe specific for the X chromosome, which hybridizes near the centromere. Two of the X chromosomes have migrated to opposite poles, but the third is arrested on the metaphase plate. The centromeres of the arrested sister chromatids have separated and remain attached to the spindle, but the chromatids have failed to separate completely.

fer of a small region of the X chromosome that included the controlling element, from the X chromosome to chromosome 2, led to the creation of a translocation chromosome that was eliminated in embryos if it was inherited paternally. de Saint Phalle and Sullivan have now found that this translocation chromosome is eliminated because the chromosome-2-derived arms do not separate. This strikingly confirms Crouse's predictions that the controlling element acts in *cis*, that it can act at a distance, and that it can affect the behaviour of an autosome as well as the X chromosome.

Why do the sister chromatids fail to separate? de Saint Phalle and Sullivan raise the possibility that a ubiquitin-mediated proteolysis, topoisomerase II activity or phosphatase activity that may be required for sister-chromatid separation at anaphase (or the metaphase-to-anaphase transition) is modulated by the chromatin structure of the eliminated chromosomes. The controlling element may be required for the formation of this chromatin structure on the X chromosome. Moreover, the L chromosomes are lost earlier than the X chromosomes in both sexes, and the X chromosomes are lost earlier in males than in females. To explain these patterns of elimination, the authors propose that a maternal factor that is required for sister-chromatid separation is gradually used up by the eliminated chromosomes. This could mean that a common mechanism is used

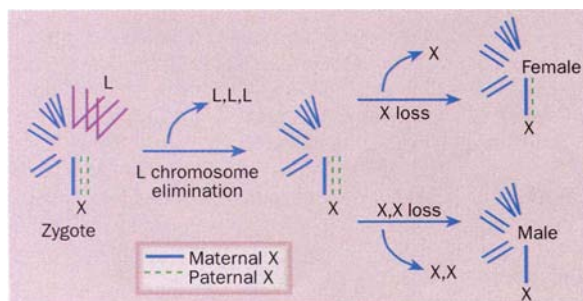


FIG. 1 Chromosome loss in early embryos of *S. coprophila*, adapted from de Saint Phalle and Sullivan<sup>1</sup>. Zygotes of both sexes have the same chromosome constitution and contain three X chromosomes — one from the oocyte and two from the sperm. The L chromosomes are eliminated first, followed by loss of the X chromosomes: the X chromosomes that are lost are always paternal in origin. Loss of one X chromosome from each cleavage nucleus results in an X/X female; the loss of two X chromosomes gives rise to an X/0 male.

to mark the L and X chromosomes for elimination, and the basis of the chromosome specificity should be the key to understanding this process.

Identification of the controlling element, its product and its targets, would be a big step forward in illuminating the mechanism of chromosome loss in the sciarid flies. This would also help us to understand the process of chromosome segregation in more 'conventional' organisms. The expectation is that *Sciara* has co-opted normal cellular proteins to achieve its unusual mode of chromosome loss and sex determination, rather than evolving a completely new system. As de Saint Phalle and Sullivan note, and as Metz pointed out previously<sup>2</sup>, the naturally occurring exceptions to the general rule — such as *Sciara* — give us a unique opportunity to investigate the mechanism of cell division. Much like the analysis

of mutants, natural variants can help to define the 'normal' process. With its programmed chromosome nondisjunction and loss during early embryogenesis, as well as the unusual behaviour of its chromosomes during spermatogenesis and paternal chromosome imprinting (not discussed here, but see refs 2, 3), *Sciara* should provide plenty of scope for further investigation. □

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## NUCLEAR PHYSICS

# Attack on a convoy of nucleons

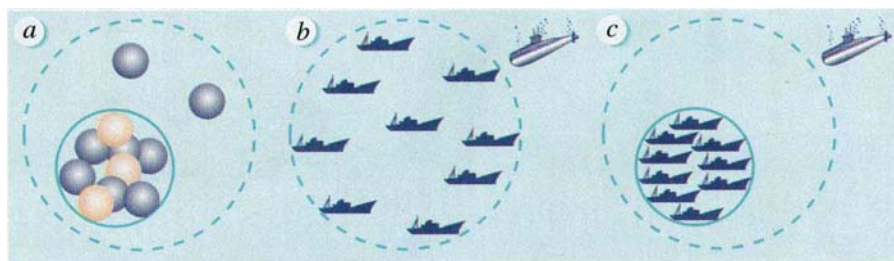
P. G. Hansen

THERE is a class of light nuclei that have diffuse 'nuclear halos' outside their central cores. Only discovered a decade ago, the phenomenon is also likely to occur in heavier nuclei, as yet not accessible to experiment. It has implications for the study of nuclear three-body effects and also for nuclear burning in stars, where the reaction rates are very sensitive to the wavefunction at large distances. Now J. S. Al-Khalili, J. A. Tostevin and I. J. Thompson of the University of Surrey have shown that the halos are considerably larger than was believed<sup>1,2</sup>.

The neutrons and protons, jointly called nucleons, that constitute an ordinary nucleus are (in their lowest-energy state) arranged in the form of a droplet, with a constant density in the interior and a sharp surface. In contrast, a halo state has a few of the nuclear particles at distances of several times the nuclear radius. Because quantum mechanics does not allow us to know more than the probability of finding a particle at a certain position, the halo particles will on average appear as a thin and blurred cloud at the exterior; a 'nuclear stratosphere'.

An example is the three-body halo system,  ${}^6\text{Li}$  (*a* in the figure), consisting of a  ${}^4\text{He}$  core coupled to two distant neutrons. The reason for the halo is the low binding energy of the neutron pair (only 0.3 MeV, compared with 10 to 30 MeV for a pair in a stable nucleus), which permits it to make excursions far from the core by quantum-mechanical tunnelling. Whereas the 'tunnel' in alpha or proton decay<sup>3</sup> is like a road tunnel, in this case it is like a mine gallery that comes to a dead end.

At a more fundamental level, the effect may be understood from the Pauli exclusion principle, which forbids two or more identical particles to occupy the same quantum state. If neutrons are added one by one to a normal nucleus,



*a*, The compact nucleus  ${}^6\text{Li}$  (three protons and six neutrons), with radius 2.3 fm, is coupled to two neutrons at an average distance of approximately 7 fm to form the  ${}^6\text{Li}$  halo system. *b*, Cargo ships in a dispersed formation and *c*, in a tight convoy (see text).

the Pauli principle forces them into states of higher and higher momentum. As a last resort, when the core becomes neutron-saturated, the nucleus squeezes most of the wavefunction of the last neutrons outside to form a halo, which because of its large size is allowed to have lower momentum.

This is an example of Heisenberg's space-momentum uncertainty principle, which also relates the two main experimental tools for detecting the halo<sup>4,5</sup>. In momentum coordinates, the halo manifests itself as a very narrow distribution of the dissociation products (neutrons and  ${}^4\text{He}$ , for example) from nuclear reactions. The second method, and the one that is the subject here, is to study the problem in spatial coordinates.

Qualitatively, the picture is simple. The reaction cross-sections for collisions of halo states with other light nuclei are larger than those for a normal structure, and the size of the halo can be inferred from this difference. But the translation from cross-section to size is not simple. At high energies and for ordinary nuclei, there is an established approach called Glauber theory that can evaluate how much the nucleons shield each other from the projectile.

This shielding can be illustrated by an example taken from naval warfare. Cargo ships sailing in a dispersed formation (*b* in the figure) are all equally exposed to submarine attack, but the attacker is unlikely to penetrate far into a dense convoy (*c*), so the proportional losses should be smaller — the 'reaction cross-section' per ship is reduced. During the Second World War, the Operations Analysis Group<sup>6</sup> of the British Admiralty found that theoretical arguments and operational statistics agreed — large convoys suffer much lower relative losses than small convoys. This led to a change of official strategy in 1943. A second factor, missing in nuclei, is that a large convoy tends to have a better-defended perimeter.

Now consider the collision of a single nucleon with a nucleus. For a hypothetical nucleus of very low density, the total cross-section is the number of particles times the average nucleon-nucleon cross-section. For a denser arrangement, the

total cross-section will be smaller because the nucleons shield each other. It is this shielding that, indirectly, conveys information about the nuclear size.

The effect is present even in the lightest nucleus, the deuteron, which consists of a neutron and a proton at an average distance of 4.4 femtometres (fm), much larger than the nucleon radius of 0.7 fm (one femtometre is  $10^{-15}$  m). Here, shielding requires that the neutron and the proton are aligned exactly in the direction of the incident beam.

For heavier nuclei, calculations start with a theoretical model of the nucleus which yields a spherically symmetric average density distribution. Then the reaction probability for a nucleon traversing the nucleus is calculated at each point of the



projected area of the target. This probability, based on the known nucleon-nucleon cross-sections, is integrated to give the total theoretical reaction cross-section, which is compared with the experimental value. If the two agree, one assumes that the nuclear size is the same as in the original model. The same approach can give nucleus-nucleus collision cross-sections.

So what went wrong when this technique was applied to collisions of halo nuclei with carbon at energies of 800 MeV per nucleon? The halo nucleus as a whole moves much faster than its constituents do internally, so the outcome of the collision reflects the 'frozen' arrangement of that instant, as in *a* in the figure. But the averaged spherically symmetric distribution is spread out, more like *b*, and therefore underestimates the screening and overestimates the reaction probability. So when the size is inferred from a measured cross-section, the halo radius is underestimated. By taking the instantaneous structure of the halo system into account, the Surrey group arrives at new estimates.

The effects are large. For  $^{11}\text{Li}$  the average radius goes from 3.10 fm to 3.53 fm, with the  $^9\text{Li}$  core radius still at 2.30 fm. This means that the neutron halo must have a radius of about 7 fm — huge compared with the core. For  $^{11}\text{Be}$ , the mean radius of the single-neutron halo increases from 5.6 fm to 6.7 fm, which more nearly agrees with shell-model predictions. The new method has the attractive feature that it treats the reactions of the composite system and of its constituents in a unified way, so the consistency of the results can be checked against experimental data for each subsystem.

The conventional calculations, with their underestimated radii, have served as theoretical anchor points for studies of halo states in nuclear structure and astrophysics, so the new results are certain to lead to a revision of many of our ideas. The most immediate impact will be on our understanding of the so-called Borromean systems, such as  $^6\text{He}$ ,  $^{11}\text{Li}$  and  $^{14}\text{Be}$ , in which the core binds two neutrons in the halo, but would not bind just one. There could also be consequences for our precise understanding of the proton halo of  $^8\text{B}$ , central to the solar neutrino puzzle. □

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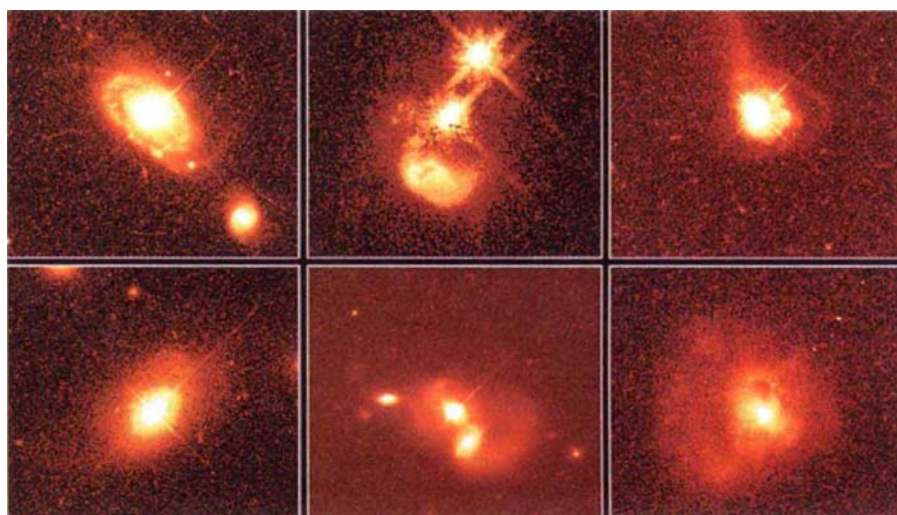
## The sharp end of quasars

*Patrick S. Osmer*

THE population of quasars evolves strongly with time. They are rare objects at present, but their space density was 1,000 times higher when the Universe was about one-fifth of its present age (at a redshift of about two). At still earlier times, the space density appears to decline steeply. Have we seen back to the time when quasars formed? Or is our view blocked by obscuring matter along the lines of sight to the great distances involved? New results reported by Shaver *et al.* on page 439 of this issue<sup>1</sup> indicate that obscuration by dust is not the cause

evolution at high redshift. Shaver *et al.* worked with 878 radio sources in an area covering 40 per cent of the sky, making use of optical sky surveys to characterize each source, and making new radio and optical observations where necessary. They were able to identify all the radio sources either as galaxies or as stars, and they conclude that none are quasars with  $z > 5$ . They did find a quasar at  $z = 4.46$ , which is the highest-redshift radio-loud quasar known.

The case for a decline in the space density of radio-loud quasars is based on finding 25 quasars with  $z < 4$  and none with



J. Balcells, M. Disney & NASA

Nearby quasars are found in normal, colliding and irregular galaxies (left, centre and right). The sharp drop in quasar numbers as we look back to near the beginning of the Universe is an important clue to how matter was assembling into galaxies and quasars at the time.

of the decline, and that we are seeing the epoch when quasars became active.

The observed evolution of quasars for redshift  $z < 2$  is strong, and has been well established since its discovery by Schmidt in 1968 (ref. 2). What happens at higher redshift has been debated for at least two decades. In recent years, technology has advanced enough to allow new quantitative surveys at optical wavelengths, showing that the space density of quasars declines beyond  $z = 3$ . But the observed visible-wavelength light was emitted at ultraviolet wavelengths and subjected to considerable absorption by intervening dust in the cosmos; and the geometry of the expanding Universe is such that dust in galaxies lying between us and distant quasars could cause the apparent decline in space density that is seen at high redshift. Surveys at other wavelengths provide an independent approach to the problem.

Cosmic dust is virtually transparent to radiation at radio wavelengths — indeed, the first quasars were identified by their radio emission. But only a small fraction of them are strong radio sources, so large samples are required to investigate their

$z > 5$ . In addition, surveys at different wavelengths show similar variation of the space density with redshift, whereas dust obscuration would be colour-dependent; and Boyle and di Matteo<sup>3</sup> came to a similar conclusion from analysis of an X-ray-selected sample of quasars, which also should be little affected by dust. So it seems that the decline is real, and we are seeing the epoch when quasar activity began.

But dust certainly does exist in galaxies, and there is evidence for it in the systems that produce quasar absorption lines. The ultraluminous infrared galaxies that have been found at low redshift (and more recently up to  $z = 4.7$ ) may be powered by nascent quasars, which have yet to burn

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through their surrounding cocoon of dust clouds<sup>4</sup>. Does this contradict the claims of Shaver *et al.*? Not necessarily, as long as such objects are rarer than optically selected quasars, and their behaviour does not change much with redshift.

What are the cosmological implications of identifying the epoch when quasar activity begins? Quasars mark high-density concentrations of matter in the Universe, tracing structure on larger scales than any other discrete objects; and they probably also mark the early formation of galaxies. So when the high-redshift limit of known quasars reached 4.9 (ref. 5), there was concern that the huge concentrations of matter needed for quasars would not have had time to form in standard models of galaxy formation. Recent theoretical results<sup>6</sup>, however, show that the familiar cold-dark-matter model can produce the needed sites for quasars, even if most galaxies assemble later on.

In the meantime, the Hubble Space Telescope and the Keck 10-m telescope

have opened new windows on the high-redshift Universe by observing galaxies out to  $z > 4.5$ , thereby presenting opportunities to study directly the relation of quasars and galaxies at their epoch of formation. With the rapid decrease in the space density of quasars at high redshift, starburst galaxies may soon replace quasars as the objects of largest known redshift.

The theory of galaxy and structure formation is advancing as dramatically as our observational capabilities<sup>7</sup>, and the latest results indicate that stars are already forming by  $z = 6$ , as dense clumps of gas build up in the early stages of galaxy formation (D. Weinberg, personal communication). If advances in observations and theory continue at their present rate, we may find that the end of quasars has led us to the beginning of galaxies. □

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## DEVELOPMENTAL NEUROBIOLOGY

# Netrins find their receptor

Uwe Drescher

WHAT guides migrating nerve axons to their correct targets during development? This is one of the basic issues in developmental neurobiology, and chemoattraction has long been thought to be involved. A tangible answer began to emerge with the demonstration that explants of rat spinal floorplate promoted the outgrowth of axons from commissural neurons, and caused these axons to turn<sup>1,2</sup>. Subsequently, the specific factors concerned in axon turning were identified<sup>3-6</sup>. These factors, called netrin-1 and netrin-2, are phylogenetically conserved molecules related to the product of the *unc-6* gene in *Caenorhabditis elegans*.

Netrins have since been in search of a receptor. As reported in *Cell*, however, three groups have now cloned it from *C. elegans*<sup>7</sup>, rat<sup>8</sup> and *Drosophila*<sup>9</sup>. The nearly simultaneous identification of the homologous receptor in three different species reflects both the intense interest in axon guidance and the remarkable degree of conservation of this system in organisms that have such different evolutionary histories.

The netrins are secreted molecules that are related to the extracellular matrix protein laminin, and they are expressed in floorplate cells and in the ventral two-thirds of the vertebrate spinal cord, respectively<sup>4,5</sup> (see figure). The floorplate, a structure comprising the cells occupying the ventral midline of the spinal cord, is an intermediate target for commissural axons, which come from cell bodies located in the dorsal half of the spinal cord<sup>10</sup>. It is

thought that a ventral-to-dorsal gradient of netrin protein is interpreted by migrating cells or axons as either a repulsive or an attractive guidance cue, depending on their receptor composition. In the first case, axons would grow dorsally away from higher concentrations of netrins, as in the case of certain subclasses of motor neurons; in the second, they would grow ventrally towards higher concentrations, as with commissural axons.

Work with *C. elegans* previously revealed that mutations to three genes — *unc-6* (a netrin homologue), *unc-5* and *unc-40* — all specifically perturb dorsoventral migration of pioneer axons and cells<sup>11</sup>. The interplay between these genes is complex. Mutations in *unc-5* and *unc-6* disrupt dorsal migrations, including those of the major classes of motor axons; *unc-40* mutations cause comparatively mild defects in dorsal migrations. Mutations in *unc-6* and *unc-40* also disrupt ventral migrations, including those of several classes of sensory and motor axons. In all migrations that depend on *unc-6*, expression of *unc-40* is required for ventral migration. Functional *unc-40* is also required for some *unc-6*-independent movements<sup>11</sup>.

As described in the first of the new papers, Chan *et al.*<sup>7</sup> have used positional cloning to identify the *unc-40* gene. Surprisingly, it turns out to encode a product that is closely related to the vertebrate proteins Deleted in colorectal cancer (DCC)<sup>12</sup> and neogenin<sup>13</sup>. Like UNC-5 protein<sup>14</sup>, UNC-40 is a member of the

immunoglobulin superfamily, but has four immunoglobulin domains and six fibronectin type-III repeats. It is expressed in those cell populations that are affected by *unc-40* (and *unc-6*) mutations. In particular, UNC-40 is found in subpopulations of motor neurons and in sensory neurons whose axons orientate towards UNC-6 sources at the ventral midline (see figure). The axons of the mechanosensory neurons designated AVM and PVM normally project towards the ventral nerve cord, but often fail to do so in *unc-40* mutants. Chan *et al.*<sup>7</sup> found that this defect could be rescued by a transgene directing expression of UNC-40 to these cells, showing that UNC-40 acts in a cell-autonomous fashion.

As to the relationship with its vertebrate homologues, UNC-40 has 30% and 26% identity in the extracellular domain with DCC and neogenin, respectively; together, these molecules form a separate subfamily within the immunoglobulin superfamily. The DCC gene<sup>12</sup> was originally identified as a candidate tumour suppressor, which is lost at high frequency in colorectal and other cancers. Earlier functional studies of DCC protein have suggested that it somehow participates in signalling pathways that regulate cell proliferation or differentiation (or both)<sup>15</sup>. Chick neogenin<sup>13</sup>, which is about 49% identical to DCC over its entire sequence, is expressed predominantly in embryonic brain. It is upregulated on subsets of neuronal cells when cell division ceases and axon outgrowth begins, then downregulated when neurite growth is completed, observations which support the idea that it is involved in axon guidance.

Given the similarity of *unc-40* to the genes encoding DCC and neogenin, Keino-Masu *et al.*<sup>8</sup> used the polymerase chain reaction to clone members of this gene family in rat, and found just the two homologues. They concentrated further analysis on DCC protein, as it, but not neogenin, is expressed on commissural axons and growth cones, which are known to be attracted by netrin/UNC-6 (see figure). Most importantly, they showed that DCC binds netrin-1 with a dissociation constant that is consistent with the effective dose for netrin-1 on commissural axons, and that an antibody to DCC could block the outgrowth-promoting effect of netrin on commissural axons.

Keino-Masu *et al.*<sup>8</sup> could not, however, determine whether DCC participates in the turning response of commissural axons towards a netrin source. The antibody to DCC did not block this response in *in vitro* assays — which could imply that DCC is not required for netrin function in this situation, that the antibody blocks only a particular function of DCC, or that the antibody simply does not penetrate the spinal cord explant *in vitro*.

In the third of the new papers, Kolodziej *et al.*<sup>9</sup> identified Frazzled pro-

## Life inside out

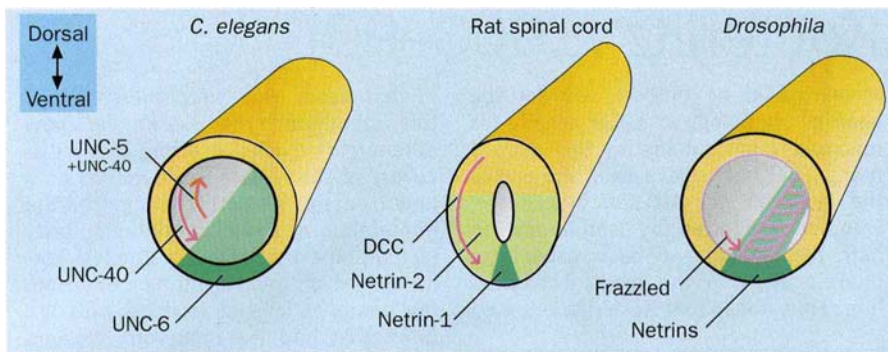
ALMOST all living things are made of cells densely packed together in a watery intercellular medium. The outer cell membrane may simply be an oily bilayer of phospholipid molecules, with various proteins swimming in it. An assembly of cells is rather like a dense emulsion of oil in water, but with a living interior in each droplet.

Now dense emulsions have a remarkable trick. They can invert. Agitation, or chemical additives, can flip a dense emulsion of oil in water over into a dilute one of water in oil. The densely packed oil drops merge into a continuous phase, isolating the water phase as little drops within the oil. So Daedalus wants to invert living tissue. The result would have all the cells merged into one continuous phase, with the intercellular medium dispersed within it as small droplets. Furthermore, says Daedalus, the product would still be alive and healthy. The same area of bilayer surface would separate the merged cells from their 'external' medium, despite the fact that it was now inside them. The cell phase would go on absorbing oxygen and nutrients from that medium, and dumping waste material into it. Ultimately, of course, the entrapped drops of 'external' medium would be exhausted; but for a while the tissue would not even notice its inversion.

To invert the tissue, Daedalus plans to shrink the outer monolayers of its cell membranes, encouraging them to flip from convex to concave. A sudden douche of heavy water, he argues, should do it. It will reduce the hydration energy of the outer phospholipid layer, thus dehydrating and therefore shrinking that layer. (Excessive heavy water is bad for many organisms, maybe for this very reason.) Ultrasonic agitation of the strained tissue should then invert it.

Daedalus wants to try it on cultured tissue first. The obvious experiment on an inverted tissue is to dose it with plasmids; with no cell membranes to penetrate, they will diffuse freely through the continuous multi-nucleate mass. But fast work will be needed. The droplets of intercellular medium within that mass will rapidly lose their heavy water and nutrients by outward diffusion. Soon it will be time to apply a blast of fresh heavy water and ultrasonics, and invert the tissue back again.

It will be somewhat disorganized. As the continuous mass repartitions into cells again, some will enclose two or more nuclei or other organelles, while others have none. Many will die, but the survivors will teach surprising and instructive lessons. Biology will take another step forward. David Jones



Cross-sections through *C. elegans*, rat spinal cord and *Drosophila* at early stages of development. The diagram shows the expression pattern of netrin receptors on various axonal trajectories, in relation to ventrally expressed netrins, as identified by Chan *et al.*<sup>7</sup>, Keino-Masu *et al.*<sup>8</sup> and Kolodziej *et al.*<sup>9</sup>.

tein, the *Drosophila* homologue of UNC-40, by using an enhancer trap screen for mutations affecting the nervous system. In the *frazzled* mutant, posterior and, to a lesser extent, anterior commissures in abdominal segments are thinner or are absent, and breaks in the longitudinal tracts are occasionally observed. This phenotype closely resembles that seen for null mutations of the two *Drosophila* netrin genes<sup>16,17</sup>, which parallels the similarity of the *unc-6* and *unc-40* mutant phenotypes<sup>11</sup>.

In wild-type flies, Frazzled protein is expressed at high levels on commissural and longitudinal axons in the developing central nervous system (see figure), and at lower levels on peripheral motor axons that extend outward in the intersegmental and segmental nerves. It exists in two isoforms, which differ in an insertion of about 150 amino acids between the immunoglobulin and fibronectin type-III repeats. Overexpression of the shorter Frazzled isoform rescues some but not all mutant phenotypes, suggesting that the longer isoform has some distinct functions. Mutants of *frazzled* also have defects in intersegmental nerves. These project normally towards netrin-expressing muscles, but they wander into adjacent segments and make contact with inappropriate muscles, implying that Frazzled has an additional function in target recognition.

Taken together, the three new papers<sup>7-9</sup> constitute a considerable step forward. But inevitably the picture may be more complicated than it might seem, and other molecules besides those cloned so far may well be involved in netrin signalling. For example, DCC is expressed on spinal motor neurons, which are not sensitive to netrin. So DCC alone is not sufficient to confer responsiveness to netrin, and there may be a requirement for an additional molecule (or molecules) that is expressed on commissural but not spinal motor axons<sup>8</sup>. On motor neurons, DCC could interact with a different ligand or be antagonized by unknown downstream signalling molecules.

Alternatively, the netrin receptor may be heteromeric — studies on *C. elegans*

show that some dorsal migrations have a dual requirement for UNC-5 and UNC-40 (ref. 11), and the phenotype of *unc-40* null mutations is less severe than those resulting from mutations to other alleles which result in truncated versions of UNC-40 protein<sup>7</sup>. So the truncated forms may act as dominant negatives by inhibiting a normal binding partner of UNC-40.

Finally, because DCC is expressed at very low levels on trochlear motor neurons<sup>8</sup>, which are repelled by netrin-1 (ref. 18), there may be other receptors for netrin. One candidate might be an UNC-5 homologue, because UNC-5 is involved in dorsally directed movements of axons in *C. elegans*.

It was Ramón y Cajal who, more than a century ago, proposed<sup>19</sup> that migrating axons are guided to their destinations by chemoattractants. This latest work paves the way for the identification of co-receptors for netrins, and other downstream molecules, and for a modern, molecular understanding of his idea. □

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# Hans Kosterlitz (1903–96)

HANS Kosterlitz, who died on 26 October in his beloved Aberdeen, often remarked that he had really wanted to be a mathematician but that medicine offered a more certain living. His mathematical grasp of problems stood him in good stead, however, over a scientific career which spanned more than 50 years, and which culminated in the development of a quantitative *in vitro* bioassay for morphine and its congeners and its use to discover the brain's own 'opiates', the enkephalin peptides.

Pharmacologists have always claimed bioassay as their own, and it was perhaps ironic that one of its most triumphant applications should come at a time when the new receptor-binding assay technology was changing the face of pharmacology. The race to isolate and identify the opioid peptide enkephalins was eventually a contest between bioassay as practised by Kosterlitz's group at the University of Aberdeen, and opioid-receptor binding, originally developed by the groups of Avram Goldstein, Eric Simon, Lars Terenius and Solomon Snyder, and vigorously exploited by Snyder. But the discovery and its impact on neurobiology was much more than a triumph of technique — it was rooted in assiduous scholarship and the careful testing of hypotheses arrived at by reason and experiment, virtues that Hans passed on to generations of students and colleagues.

Although not a Jew himself, Kosterlitz foresaw where Nazi policies were leading and left his clinical assistantship at the University of Berlin in 1933 to work with J. J. R. Macleod in the Department of Physiology at Aberdeen. Macleod, who had shared the Nobel prize for the discovery of insulin, had offered Kosterlitz the opportunity to pursue his interest in liver glycogenesis and the role of insulin.

Almost immediately, however, with the unexpected death of Macleod in 1935, Kosterlitz was faced with a dilemma — should he stay on in Aberdeen without a mentor or move south to Oxford? He chose to stay, a decision which in private he sometimes regretted. Aberdeen at that time was rather insular and it could be difficult for foreigners (which included anyone from south of the River Tay). Kosterlitz's abilities were quickly recognized, however, and he coped with any xenophobia by becoming more Scottish than the Scots, not least in his love of the country's culinary products.

Nevertheless, his academic progression was slow, and he was bitterly dis-

appointed to be passed over for the chair of physiology in 1959 despite his manifold contributions to the department. That might have been the end of the story but for Alastair MacGregor, who had recognized the need to develop both the clinical and basic aspects of pharmacology in the medical curriculum. Thus it was that Kosterlitz returned

to a clinical department as professor of pharmacology at the age of 65.

Kosterlitz continued to work on the glycolytic pathways after Macleod's death and succeeded in isolating galactose-1-phosphate as an intermediary metabolite. This might have received greater recognition if he had gone to Oxford. But it was not to be; the war intervened and Kosterlitz took up nutritional research to support the war effort. This not very exciting period of his career ended in the 1950s with a sabbatical period at Harvard with Otto Kraye to work on the effect of the veratrum alkaloids on the autonomic nervous system.

This change in direction was typical of Kosterlitz, who had recognized the growth of autonomic pharmacology and its potential. His research on gut reflexes led him inexorably to study the effects of morphine, which had been reported by Paul Trendelenburg to inhibit myenteric reflexes in the ileum. Kosterlitz refined this observation into a quantitative assay, involving the guinea-pig longitudinal muscle-myenteric plexus, and with Louise Cowie and Angela Waterfield he showed that the analgesic activity of a wide variety of opiates could be predicted from their activity in this bioassay.

When, in 1969, I joined Kosterlitz in Aberdeen, the interest in opiates was intense. MacGregor affectionately called us the cat doctors and it was the cat nictitating membrane which brought our two lines of research together. Together with Graeme Henderson, we showed that noradrenergic transmission

in that tissue was morphine-sensitive; this stimulated the search for new adrenergic models, resulting in the discovery of the mouse vas deferens as a new bioassay. Kosterlitz recognized the importance of reducing the complexity of morphine action to its simplest elements, which meant getting away from the complications of working with the brain that had led numerous workers into unproductive paths.

Our group established without doubt that morphine and its congeners acted as agonists at stereoselective receptors to modulate neurotransmission. When all other possibilities had been eliminated, the inference was clear — some undiscovered neurochemical system must use an endogenous opiate. It has been assumed that the discovery of opiate-receptor-binding sites in 1973 played an important role in establishing this hypothesis. But it was only a confirmatory one, because Kosterlitz and I had already agreed on the endogenous opiate hypothesis in 1972 and made plans to test it in the Unit for Research on Addictive Drugs formed upon Kosterlitz's retirement in 1973.

For 12 years this unit was an international leader in opiate research. Discovery of the enkephalins and other opioid peptides opened up the new era for neuropeptides, whose physiological and therapeutic implications are still being vigorously explored. The rigorous classification of the  $\kappa$ -opioid receptor, for instance, and the discovery of the  $\delta$ -opioid receptor, opened up new therapeutic avenues.

Kosterlitz won widespread recognition and many honours, including a Lasker Award in 1978, for these and other seminal studies. It was controversy over the Lasker, awarded at the same time to Snyder and myself, that perhaps deterred the Nobel committee from recognizing the opiate work. A student of Snyder claimed that she should have shared the award. Kosterlitz and I found the incident distasteful, and supported Snyder, who has continued to make major contributions to neuroscience.

Hans Kosterlitz could sometimes appear fierce and intimidating, but those who chose to meet him on equal intellectual grounds usually found a colleague and friend for life. Hans was a physician, scientist, teacher, *bon viveur* and friend — he will be sorely missed, but his work will endure. John Hughes

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Dr Robert Stepney/Science Photo Library

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REASONS

# Spider silk as mechanical lifeline

SIR — Here I look at the relationship between the mechanical properties of silk drag-lines and a spider's weight, to explain the measured values of the mechanical strength of drag-lines. Spider silks have been studied from the points of view of mechanical strength<sup>1-3</sup>, strain energy<sup>4</sup>, physico-chemical<sup>5</sup>, thermal<sup>6</sup> and optical<sup>7</sup> properties, and ageing<sup>8</sup>. There have been many estimates of breaking strength, elastic modulus<sup>9,10</sup> and elongation from stress-strain curves. Mechanical strength has been explained at the molecular level and examined from the point of view of molecular orientation<sup>11,12</sup>.

Drag-lines secreted from the spider's body are a tool for the animal to move, fall from trees, capture insects and build orb-webs, as well as to support her weight and keep her alive. It is well known that drag-lines are a lifeline for spiders, as these animals are often surrounded by danger. The mechanical strength of drag-lines may be related to the spider's weight because spiders hang from them, but, to my knowledge, there have been no reports detailing this relationship. I therefore considered the mechanical strength of the drag-line as a 'safety coefficient'. The spider's 400-million-year evolution should have allowed the mechanical properties of drag-lines to become maximally efficient.

It is important to know whether measured values of elastic-limit strength and breaking strength are meaningful as safety coefficients. A clear relationship between mechanical strength and spider weight would provide a basis for the safety coefficient — a measure of security, equivalent to that used in structures such as lifts,

string, rope, aeroplanes, bridges and fibres.

The silk threads used in the present study were drag-lines secreted by female *Nephila clavata* when falling from a wooden bar. I carefully measured stress-strain curves for the drag-lines using a Tensilon. I defined the elastic-limit strength as the stress at which the stress-strain behaviour changes from linear to nonlinear, and the breaking strength as the stress at the breaking point. Figure 1a shows the stress-strain curve for the drag-line of a spider with a weight of  $4.6 \times 10^{-3}$  N, measured at a stretching velocity of  $3.3 \times 10^{-4}$  m s<sup>-1</sup>. I repeated the measurements four times for each sample, and averaged the data. All measurements were carried out at 25 °C and 60% relative humidity.

I plotted the elastic-limit strengths at a stretching velocity of  $3.3 \times 10^{-4}$  m s<sup>-1</sup> for *N. clavata* drag-lines against the spider's weight *W* in Fig. 1b. The elastic-limit strength increases linearly with increasing spider weight, with a slope of about 2. The stress-strain curve of drag-lines enters a nonlinear region when the stress exceeds about twice the spider's weight. This means that it is safe to use a single drag-line to carry her weight and a prey item, as long as the total stress does not exceed twice her weight. A drag-line from the spider's body consists of double filaments<sup>13</sup>. Even when one filament is broken, the other will easily support her weight. The spider's weight will correspond to the elastic strength for the single filament. Thus, elastic-limit strength gives a minimum safety and a maximum efficiency for supporting the spider's weight. Figure 1b also shows the breaking strength of *N. clavata*

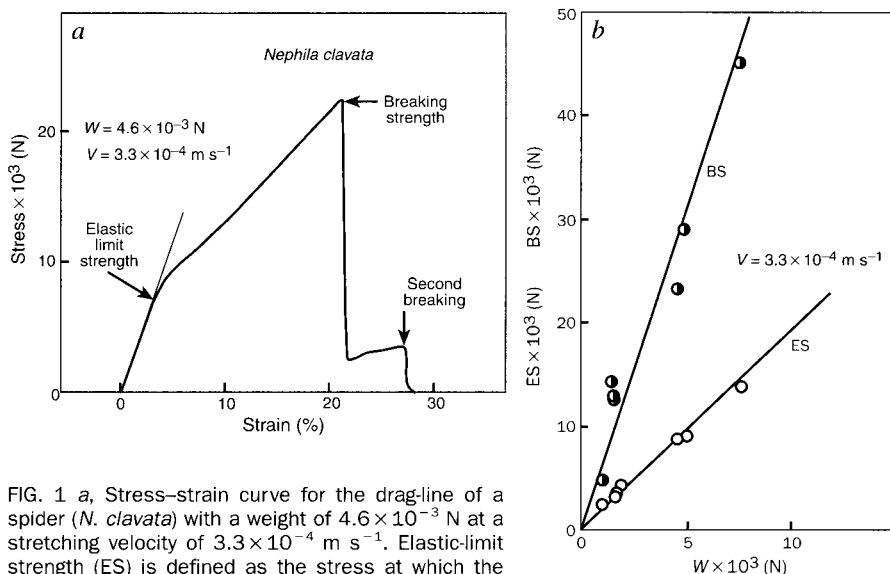


FIG. 1 a, Stress-strain curve for the drag-line of a spider (*N. clavata*) with a weight of  $4.6 \times 10^{-3}$  N at a stretching velocity of  $3.3 \times 10^{-4}$  m s<sup>-1</sup>. Elastic-limit strength (ES) is defined as the stress at which the stress-strain curve alters from linear to nonlinear. Breaking strength (BS) is defined as the stress at the breaking point of drag-lines. b, ES and BS for *N. clavata* drag-lines plotted against *W*. Stretching velocity, *V*, is  $3.3 \times 10^{-4}$  m s<sup>-1</sup>.



FIG. 2 *Nephila clavata* hanging from a silk drag-line.

drag-lines plotted against weight. The breaking strength increases linearly with increasing spider weight with a slope of about 6. Thus, drag-lines should break at a stress equivalent to that produced by about six times the spider's weight.

Because spiders move, jump and fall rapidly, their life is surrounded with various dangers. In such circumstances, maximum efficiency is needed for the mechanical strength of drag-lines. The results described here provide an estimate of this efficiency.

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## Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. Priority will be given to letters of fewer than 500 words and 10 references.



# Wide diversity of Crenarchaeota

**SIR** — The traditional perception of the diversity of the bacteria-like Archaea based on cultivation studies has been that the kingdom Euryarchaeota is a physiologically variable group including halophiles, thermophiles and methanogens, whereas the kingdom Crenarchaeota is more homogeneous, consisting exclusively of sulphur-dependent, extreme thermophiles<sup>1</sup>. This has led to the notion that the Crenarchaeota are not widespread, but are restricted to specialized habitats. Using sequence-based techniques that sidestep the cultivation of organisms, crenarchaeal small-subunit ribosomal RNA (rRNA) genes have been detected in open marine waters<sup>2,3</sup>. We now report the detection, in terrestrial lake and marsh sediments, of three novel, deeply divergent lineages of low-temperature Crenarchaeota, one of which includes the marine types. This finding indicates that such organisms are globally distributed and have an important role in the biosphere.

We isolated DNA directly from shallow-

sediment and marsh samples (5–32 °C) collected from Lakes Griffy and Lemon near Bloomington, Indiana, and used it as template in the polymerase chain reaction (PCR) to amplify small-subunit rRNA genes (rDNA), as previously described<sup>4</sup>. We used an Archaea-specific forward primer (4Fa, see figure legend) and a universal reverse primer with a polylinker tail (1492RPL) for initial amplification to enrich for archaeal rRNA genes, and then used forward primers specific to marine crenarchaeal sequences<sup>2,5</sup> (89F), or to all known Crenarchaeota (542F), in conjunction with 1492RPL to reamplify the initial PCR products. We cloned and screened the rDNA copies from both rounds of PCR by restriction fragment length polymorphism (RFLP) analysis of rDNA inserts, and sequenced a representative of each RFLP type either partially or completely (~500 or 1,500 nucleotides, respectively).

On phylogenetic analysis, summarized in the figure, we found that the collection

a high-temperature ancestry for the low-temperature organisms<sup>8,9</sup>. This correlation also suggests that the ability to grow at low temperatures has arisen within the Crenarchaeota in at least three separate instances.

The occurrence of diverse low-temperature Crenarchaeota in marine and terrestrial environments is unexpected, and shows that members of this kingdom of the phylogenetic domain Archaea are phenotypically more varied than was previously thought. Because neither these organisms nor their close, high-temperature relatives have been cultivated, we have no information about their metabolic properties. Considering the general metabolic capacities of other Archaea, however, we predict that the low-temperature Crenarchaeota can use molecular hydrogen as an energy source. The oxidation of hydrogen, generated abiotically and biotically, is a significant theme in microbial ecosystems<sup>10</sup>. The ubiquity of low-temperature Crenarchaeota in marine and terrestrial environments suggests that they are widely distributed in nature. The rRNA sequences provide tools for the study of their distribution and role in the global ecosystem.

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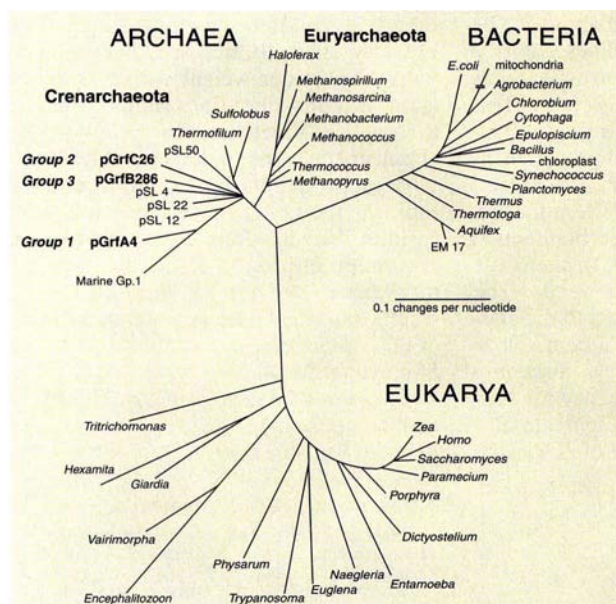
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Phylogenetic analysis of terrestrial Crenarchaeota. DNA was purified directly from sediments collected from Lakes Griffy and Lemon, and used for PCR as outlined in the text and detailed previously<sup>4</sup>. Forward primers used were: 89F, 5'-GGCTCAGTAACG-CGTAGTC-3'; 542F, 5'-CGCGGTAATACCAGCYC-3'; and 4Fa, 5'-TCC-CGGTTGATCCTGCCRG-3'. All PCR products were generated using the universal reverse primer 1492RPL, 5'-GGCTCGAGCGGC-CGCCGGGTTACCTTGTTACGACTT-3'. PCR products were cloned as described, screened for unique types by restriction analysis and unique types were fully or partially sequenced. Sequences were aligned with others from the rRNA sequence database<sup>11</sup> and phylogenetic analyses were done. The phylogenetic tree shown is a composite tree based on evolutionary distances<sup>12</sup> and full sequences of representatives of the three clades discussed in the text. Clones pGrfA4, pGrfC26 and pGrfB286 represent groups 1, 2 and 3 Crenarchaeota, respectively. All sequences determined (31 partial and full sequences) have been deposited in the GenBank database, accession numbers U59968–U59999. Sequence alignments and bootstrap analyses of phylogenetic trees were as previously described<sup>4</sup>. Additional details of methods are available electronically (<http://crab2.berkeley.edu/~pacelab/cren.html>).

describes three deeply divergent clades within the Crenarchaeota. We termed the three clades of low-temperature Crenarchaeota group 1 (17 different sequences detected, represented by pGrfA4 in the figure), which includes the marine sequences<sup>2,5</sup> and two short (200-nucleotide) sequences encountered in a survey of DNA from soils<sup>6</sup>; group 2 (14 novel sequences detected, represented by pGrfC26); and group 3 (one instance detected, pGrfB286). Groups 2 and 3 Crenarchaeota have not been detected previously. Low bootstrap values (not shown) for specific placement of the pGrfB286 line (group 3) indicate that its branch-point in the tree is not resolved, however, this lineage clearly is highly divergent.

Each of the three new clades detected in this survey is specifically associated with sequences originally cloned from Obsidian Pool, a hydrothermal (73–93 °C) spring in Yellowstone National Park<sup>4,7</sup>. The nesting of the low-temperature sequences within groups from hot springs is consistent with

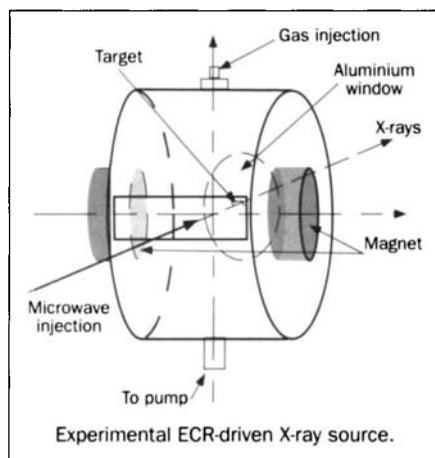
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## A compact radiological X-ray source

**SIR**—The unexpected observation we report here is that a compact electron cyclotron resonance magnetic mirror discharge can possibly produce medical X-rays with energy up to 100 keV. This device is driven by a c.w. 2.45-GHz microwave source of maximum power 700 W. It has no electron-emitting cathode and no high-voltage power supply, as required in existing X-ray tubes.

Energetic electrons in the 100-keV range, in magnetic mirrors heated by electron cyclotron resonance (ECR), were



originally observed in the field of fusion research<sup>1,2</sup>. The mechanism of acceleration of these electrons is not fully understood<sup>3-5</sup>, because adiabatic invariance theoretically predicts the electron energy to be limited to lower values. These earlier devices were much larger than the device described here (the distance between the mirrors reported in ref. 2 was 10 times larger than in the present device). It was argued previously<sup>2</sup> that large devices are required to generate high-energy electrons, or the adiabatic invariance would no longer hold.

In our device (see figure), all the parameters except the radius of curvature of magnetic force lines are the same as in ref. 2. Nevertheless, we find that the mean electron energy can be ten times higher than predicted by the scaling law reported there.

We investigated the X-rays emitted from an ECR-driven magnetic mirror formed by two circular permanent magnets (5,000 G at the surface), located at a distance of less than 10 cm from each other. The microwaves are injected at the mid-plane across the mirror magnetic field, as in ref. 1. The mirror ratio is 2.4, the resonance zones ( $B=875$  G) being located a few centimetres from the mirror axis. A small tantalum target, placed in the mid-plane, a few centimetres from the axis in the resonance zone, emits X-rays when hit by energetic electrons. A thin aluminium window filters the X-rays on the way out of the chamber.

We recorded the X-ray spectrum, as

usual, by a multichannel analyser equipped with a NaI detector. The experiments showed that in argon, the X-ray production is maximum at a pressure of  $3 \times 10^{-5}$  torr. At this pressure the tantalum plate becomes incandescent. The production of X-rays coincides with the heating of the tantalum target. This may indicate that electron acceleration is possible only at low pressure, and is inhibited by the occurrence of collisions.

Attempts to produce X-rays without the metal target indicated that the bremsstrahlung of the argon gas is not the origin of the X-rays observed under these conditions. Rather, the X-ray emission occurred from the chamber metal walls, adjacent to the magnets. This may be the consequence of the non-optimal configuration of the magnetic field, compared with that reported in ref. 2. There exists the possibility for optimization, which will enable the fluxes required for medical radiology to be achieved.

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## Deep-breathing cephalopods?

**SIR**—Madan and Wells<sup>1</sup> have explored the relationship between gill area and habitat depth. They suggest that the large gill areas found in some pelagic cephalopods enable them to cope with the hypoxic conditions present throughout much of the deep sea. This conclusion is not surprising as enlarged gills as an adaptation to low oxygen have been observed previously for deep-living cephalopods<sup>2</sup>. Furthermore, Madan and Wells make broad comparisons without resolving confounding variables such as body mass, ecology, depth and ambient oxygen, as well as asserting that fish cannot tolerate hypoxic conditions, giving cephalopods a "competitive edge" over fish. But there is a considerable literature demonstrating the existence of fish in very low oxygen and, in many cases, specific adaptations of fish to low oxygen.

Both gill area and gill diffusion capacity, as presented by Madan and Wells<sup>1</sup>, are highly correlated with body weight ( $r^2=0.51$  and  $0.73$ , respectively). The authors fail to mention, though, that gill diffusion capacities sometimes scale at less than direct proportionality<sup>3</sup>. Benthic animals can be expected to have lower metabolic rates<sup>4</sup> and perhaps lower gill diffusion capacities than pelagic species. Removal of the two benthic octopods (also the largest animals in the study) from the analysis solves much of the scaling problem. With the benthic octopods excluded, however, there is no significant difference between the deep- and shallow-living groups in either gill area (Mann-Whitney,  $P=0.24$ ) or gill diffusion capacity (Mann-Whitney,  $P=0.56$ ).

Madan and Wells state that gill areas are reduced in deeper-living fish and that fish cannot tolerate hypoxic conditions. In some cases the gills of deeper-living fishes are indeed reduced relative to shallower species<sup>5</sup>. Reduced gills in deeper-living benthic octopods have also been observed<sup>6</sup>. The phenomenon could be due to reduced activity in the deep sea. Fishes and cephalopods have lower metabolic rates at depth (ref. 4; B. A. S., manuscript submitted). However, both fish and cephalopods living within the oxygen minimum layer in the eastern Pacific (where oxygen falls to  $0.25 \text{ ml l}^{-1}$ , 10% of that cited in ref. 1) have been shown to have enlarged gills<sup>2,7</sup>. Lowered critical oxygen partial pressures, enhanced affinity of respiratory proteins and decreased activity are also demonstrated adaptations of fish to low oxygen<sup>4,8,9</sup>. Fish biomass is typically more than twice that of cephalopods at all depths<sup>10</sup>. The large biomass reported in ref. 1 is based only on total consumption of cephalopods by predators and does not have a volume component<sup>11</sup>. Not only are fishes present within the oxygen minimum layer, but they are also apparently 'out-competing' cephalopods.

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## 'Distorted' RNA helix recognition

SIR — Ibba and Söll have proposed a resolution for an apparent controversy over the basis for recognition of a critical G–U wobble base pair in an RNA helix<sup>1</sup>. We wish to point out that their proposal is not supported by extensive published literature and that more plausible explanations are available.

The controversy has surrounded the question of how the G–U pair at the third position in the acceptor helix of alanine transfer (t)RNAs is recognized by its cognate synthetase. *In vitro* experiments with a multitude of natural bases and non-natural base analogues narrowed down the functional site to the free 2-amino group contributed by G of the G–U pair<sup>2,4</sup>. (Unlike the G–C Watson–Crick pair, the G–U wobble pair places the 2-amino group of G in the RNA minor groove as an unpaired functional group.) These experiments included a demonstration that simple removal of the 2-amino group (using an I–U pair), with no other change, was sufficient to abolish aminoacylation with alanine. Restoration of the free 2-amino group by substitution of a non-natural base pair (2-amino adenosine paired with isocytidine) restored aminoacylation with alanine<sup>4</sup>.

Non-natural base analogues cannot be used in experiments that attempt to assess the state of alanylation *in vivo*. However, substitution for G–U of mismatched base pairs (such as A–C) is possible and, when used in specially constructed genetic strains, some of these substitutions have created molecules that are charged *in vivo* with alanine<sup>5</sup>. Because some of these constructions lack the free 2-amino group of the G–U pair, McClain and co-workers suggest that, in these instances, the signal for alanylation is due to indirect recognition of a distortion of the RNA helix<sup>5</sup>. They propose that indirect recognition of a distorted helix accounts for recognition of the G–U pair as well.

Ibba and Söll speculate that differences

in conditions used in the *in vivo* and *in vitro* experiments can explain the different results<sup>1</sup>. They suggest that, because the concentrations of alanine used in the *in vitro* experiments were lower than those present *in vivo*, the *in vitro* studies would miss an effect on aminoacylation of a linkage between the tRNA and amino-acid interactions that could enhance aminoacylation at higher amino-acid concentrations.

Their proposal is mostly based on their studies of glutamyl-tRNA synthetase, a class I tRNA synthetase that requires tRNA<sup>Gln</sup> as a cofactor for amino-acid activation. However, in contrast to glutamyl-tRNA synthetase, alanyl-tRNA synthetase is a class II enzyme which can activate its amino acid in the absence of tRNA. Indeed, no significant linkage between tRNA and alanine has been seen when concentrations of alanine and tRNA are varied during *in vitro* aminoacylation experiments, even at concentrations of the amino acid that match those seen *in vivo* (estimated to be about 200  $\mu$ M)<sup>6,7</sup>. Although a small effect of alanine on the  $K_m$  for tRNA was observed<sup>8</sup>, this effect was opposite to that required by the proposal of Ibba and Söll.

Ibba and Söll also imply that, if higher amino-acid concentrations were used, mutant substrates containing, for example, the I–U pair would be charged with alanine. But we find that even high alanine concentrations do not result in charging of non-G–U-containing substrates (P. Buening, K. M.-F. and P. S., unpublished data). Nor does the use of Michaelis–Menten or non-Michaelis–Menten conditions make any difference. The problem with those inactive position 3–70-mutant RNAs that have been further characterized (including the I–U mutant) is that they do not detectably bind to the synthetase, in the presence or absence of ATP and alanine<sup>2,8,9</sup>. The disruption of the synthetase-binding interaction is itself sufficient to explain the inactivity of these mutant RNAs.

The question, therefore, is whether the charging of tRNAs with mismatched base pairs *in vivo* reveals anything about charging of the wild-type substrate that has a G–U pair. McClain and co-workers consider that the inactive G3–C70-containing substrates have normal, non-distorted helical stems and, for that reason, are not charged<sup>5</sup>. However, a G3–C70 containing alanine tRNA (with an operationally 'undistorted' helical stem) is made active *in vivo* if a distal mutation is present in the anticodon stem — far removed from, and in a different domain from, where the G–U pair is located<sup>10</sup>. This variant illustrates how a new, serendipitous protein–RNA interaction can be recruited to compensate for a missing normal interaction. At face value, it says nothing about how G–U is normally recognized.

This consideration, together with the known tendency of *in vivo* measurements

of charging to distort or amplify weak signals that are difficult to detect *in vitro*<sup>1,11</sup>, and the relatively minor differences found by nuclear magnetic resonance between the active G–U and inactive I–U helices<sup>12</sup>, suggest to us that the charging *in vivo* of substrates with mismatched base pairs should be interpreted with great caution.

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IBBA AND SÖLL REPLY — Schimmel and Musier-Forsyth raise various relevant points, the most pertinent of which is the requirement of tRNA for aminoacyl-adenylate formation by glutamyl-tRNA synthetase, but not by alanyl-tRNA synthetase. This, we fully accept, makes extrapolation of results from one synthetase to the other difficult. However, it was never our intention to question the validity of their earlier results. Rather, we had hoped to put forward a model that could account for the discrepancy between the existing *in vivo* and *in vitro* data.

As Schimmel and Musier-Forsyth rightly state, the charging *in vivo* of certain substrates should be interpreted with great caution, a proviso that could be extended to *in vitro* substrates. Indeed, the disparity in size between the RNA substrates themselves represents an important difference between the two experimental approaches.

*In vivo*, the role of G–U was studied in the context of a full-length tRNA molecule containing modified nucleotides, whereas *in vitro*, RNA minihelices were used which only reiterate a fragment of tRNA<sup>Ala</sup> and in some cases do not contain a stem-loop structure. Although solution studies suggest that such mini-substrates are comparable in structure to the portion of the tRNA that they mimic<sup>12</sup>, functional comparisons suggest that this may not always be the case during interaction with synthetases.

It has recently been shown<sup>13</sup>, for example, that certain base pairs in the acceptor stem of tRNA<sup>Ser</sup> make substantial contributions to aminoacylation specificity in the context of a minihelix but not a full-length tRNA. Although this is not the case for the G3–U70 base pair of tRNA<sup>Ala</sup>, whose function has been extensively documented in both RNA contexts, this example clearly demonstrates the need for caution when attempting to reconcile *in vitro* and *in vivo* data based on divergent substrates.

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# Stealing science's clothes

Dorothy Nelkin

**Conjuring Science: Scientific Symbols and Cultural Meanings in American Life.** By Christopher P. Toumey. *Rutgers University Press*: 1996. Pp. 197. \$47 (hbk); \$16.95 (pbk).

CHRISTOPHER Toumey's field, the anthropology of science, is under siege in the United States, a target in the so-called 'science wars'. Studying science as a human activity and scientists as a cultural community, anthropologists scrutinize the human dimensions of scientific work. This is a problem for some scientists who prefer the traditional view of science as a way of knowing that is free of human constraints. They have attacked the field of science studies as a post-modernist fad.

Given the heat generated by the science wars, they have been difficult for science-studies scholars to ignore. I therefore approached *Conjuring Science* with eager anticipation. How would Toumey deal with the science wars? Well, in his very first chapter, he explicitly distances himself from the debate: "I have little to say here about those post-modernist questions that ask whether science is real. The headbanging disputes that have set deconstructionists against positivists are not for me."

Toumey focuses less on science than on what science stands for in American society. How does it fit into the American democratic culture? How is science used in the context of American values? Toumey observes a remarkable contradiction between the extraordinary popular respect for scientific authority on the one hand and what he sees as antisience tendencies on the other. Americans, he says, appropriate not the content of science but its appearance. They are 'conjuring' science, invoking its images and its authority without proper comprehension, to bestow scientific credibility on commodities or ideas.

Toumey develops his argument coherently and colourfully by providing several examples of how science is appropriated in controversies. He describes disputes over fluoridation, AIDS policy, cold fusion and creationism. In each case, the key issues are not scientific but social; they include impersonal bureaucracies, the failure of governments to protect individuals, or the violation of moral or religious theories. Yet, he points out, in each controversy participants enlist science to support their causes. Moreover, in each case, science serves many different and sometimes conflicting agendas. And the meanings attributed to science may have nothing to do with its actual content.

Toumey writes well. His book is fun to read and his examples of the "looting of

science for its images and metaphors" capture the dynamics of a familiar problem. Scientific entities (the gene, for example) often assume cultural meanings that have little relationship to their scientific content. But Toumey underestimates the role of scientists themselves as conjurers — the way they cultivate the social meaning of their work.

Although Toumey tries to distance himself from "post-modernist questions", he cannot avoid them. By separating the world of scientists from the social world, he enters the "headbanging disputes", and mainly on the side of positivists. Arguing that the value of science to the public is contingent on cultural meanings, he ignores the cultural meanings imposed by scientists. Observing that people "hijack" scientific symbols, he implies that scientists value science only for its truth. But do not scientists draw moral and philosophical lessons from science that extend well beyond its content? Are not scientists engaged in conjuring when they promise miracles such as cures for genetic disease? What about the sociobiologists who draw moral and philosophical lessons from their theories? Scientists often 'hijack' religious imagery: the genome is the 'bible' or the

'book of Man'; the subatomic quark is the 'God particle'. They too are appropriating the compelling concepts of one belief system to meet the needs of another.

Toumey places blame for conjuring on sensational journalism, the popularization of weak science and the mediocre state of science education. Greater science literacy, he says, can be a solution, providing citizens with the tools for self-protection. Here he accepts the assumptions of scientists who believe that a public better informed about science would be less likely to question scientific priorities. But the problem may be less a lack of science literacy than a weakness of critical judgement. Perhaps it is literacy, not science literacy, that may encourage clear and independent thinking.

Toumey's book is interesting, creative and important, because he documents a prevalent and sometimes dangerous social tendency, the appropriation of scientific images for political and social goals. But equally important and sometimes more subtle are the similar tendencies among scientists.

That Toumey finds it necessary to dissociate himself from the postmodernist analyses that would raise such questions serves only to narrow his perspective. It also suggests that the science wars are influencing the field of science studies, intimidating those scholars who would look critically at the cultural assumptions underlying scientific work. □

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THE largest and most impressive of the Lepidoptera are silkmoths. *Wings of Paradise: The Great Saturniid Moths* by John Cody includes 72 paintings of members of this family. Above is shown the comet moth of Madagascar. University of North Carolina Press, \$60.

## How allies shared radar secrets

Frederick Seitz

**The Invention that Changed the World\*.** By Robert Buder. *Simon and Schuster, Little, Brown: 1996. Pp. 575. \$30, £18.99.*

ANYONE who has experienced a smoothly safe landing in an airliner in dense fog can offer a prayer for the wonders of modern radar. It has added a major dimension to the use of wireless during the past century. This excellent book, written in a highly readable semipopular form, describes the essentials of the historical and the technological underpinnings of the subject.

Part of the core of the book focuses, not inappropriately, on the accomplishments of the Radiation Laboratory at the Massachusetts Institute of Technology (MIT) during the Second World War, as

United Kingdom and the United States. Buder interviewed some 130 individuals while preparing the manuscript. This made it possible to add much colour to the narrative. We read of struggles, frustrations, successes and intrigues. The human aspects of the story are no less interesting and revealing than the technical ones.

Buder opens with a vivid description of the agreements reached between the leaders of the United Kingdom and the United States during the spring and summer of 1940 and following the fall of France. The British agreed to turn over to a newly created laboratory, staffed by the best scientific and engineering talent that the United States could muster, their most treasured secret knowledge concerning the great capabilities of the cavity magnetron as a powerful source of sub-metre microwaves. This was a landmark event, requiring special courage on the part of the British, albeit born of desperation, because the United States had not yet entered the war and was barely beginning to understand

mously effective pool of talent for creative work with the minimum of bureaucratic constraints. The extension of essentially the same pattern to other projects, such as those centring on the proximity-fused projectile and the nuclear bomb, proved to be equally successful. One wonders if a similar pattern of management of key programmes would emerge and be as effective today if a crisis of comparable proportions were to arise.

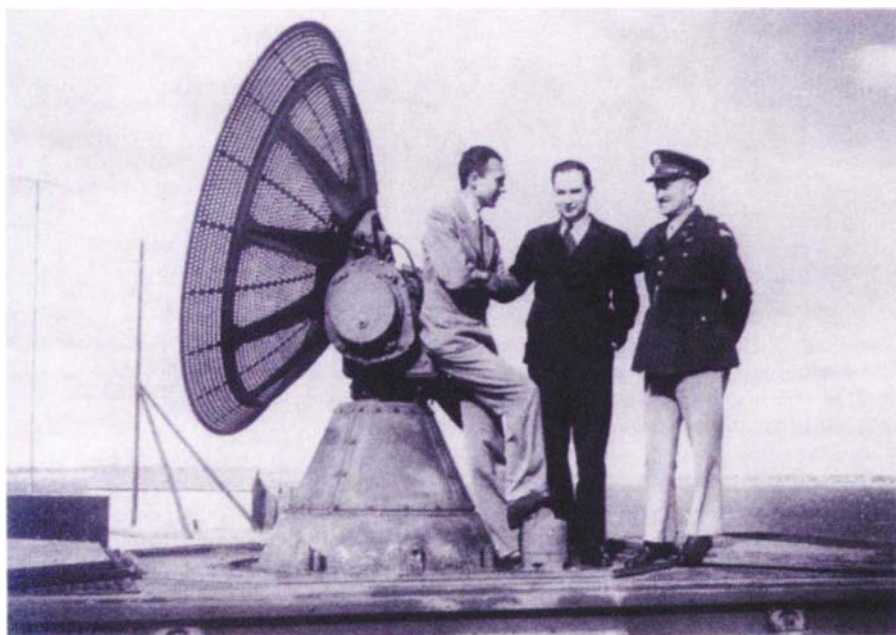
Buder has garnered much information on the German side of the radar story. Some of this material deals with the formidable offensive and defensive systems developed in Germany. But the most interesting parts focus on the numerous failures, some of which arose from overconfidence early on. Even more important was the failure of the German leaders to make any effective use of the pool of imaginative academic talent that proved so critical in the United Kingdom and the United States — assuming that the members of that pool would have been willing and able to participate on anything like a comparable scale in the twisted, police-state climate in Germany.

One of the wonders surrounding the development of radar, touched on in the author's accounts of the early history of the subject, is that it took so long to get the combination of financial and technical attention it deserved in view of the important status it finally achieved. A practical version for avoiding collisions of ships in crowded harbours and in fog was patented and tested successfully as early as 1904 when coded wireless transmission of messages was taking off. No support for further development emerged. Guglielmo Marconi urged such development in 1922 when radio operators in many countries began observing the reflections and modulations of radio waves from standing and moving metal vehicles.

Such observations stimulated the imaginations of observant scientists and engineers, but were not reflected in budgetary support. The imminent prospect of war in the mid-1930s not only provided justification for the cost of development, at long last, but also broke through the mindset, all too common in civilian and military circles, that induces hesitation to embark on great new ventures of uncertain outcome.

Almost half the book is devoted to developments since the Second World War and the uses of radar for various purposes, both civilian and military, as well as to the areas of basic science that were opened up as a result of gaining access to powerful new sources of radiation in very useful segments of the electromagnetic spectrum. This part of the book borders on the encyclopaedic and provides a broad picture of some of the most interesting advances in engineering and the physical sciences during the past half century.

Buder has carried through an ambi-



Radiation Laboratory experimental radar during anti-aircraft trials in 1942.

well as matters derived from that endeavour. But the book also pays dutiful attention to the seminal and heroically creative work in the United Kingdom that provided the foundation for the Radiation Laboratory. Robert Buder also gives good accounts of earlier research that generated interest in the development of radar for military purposes and of the great influence of microwave research on many fields of basic scientific research in the postwar period.

Not least, the book does not fail to focus on the activities of numerous participants in critical aspects of the development of radar, particularly those in the

the importance of introducing rigid security measures involving civilians.

Also of importance for the conduct of much of the scientific endeavour during the rest of the war was the understanding, perhaps never formally expressed, that the Radiation Laboratory at MIT would be primarily a civilian rather than a military-led enterprise and that most of the leading recruits would come from the academic world. That pattern had proved to be the most effective in the United Kingdom in its time of dire need. The leadership in the White House was prepared to adopt the same design for the start of a major enterprise. It would be difficult to overstate the importance of that step because it unleashed an enor-

\*UK title is *The Weapon That Won the War*.



tious enterprise very well. The book merits a broad readership and should endure as a semipopular classic in the scientific and technical literature. If I were asked to express a disappointment, it would lie in the fact that I found no mention of the hand-carried transport from France to England of the highly advanced French cavity magnetron by Maurice Ponte, just as Paris was falling in May 1940. This probably ranks on a par with the transfer of highly secret British technology to the United States a few months later. □

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## Rubbing shoulders with giants

Graham Farmelo

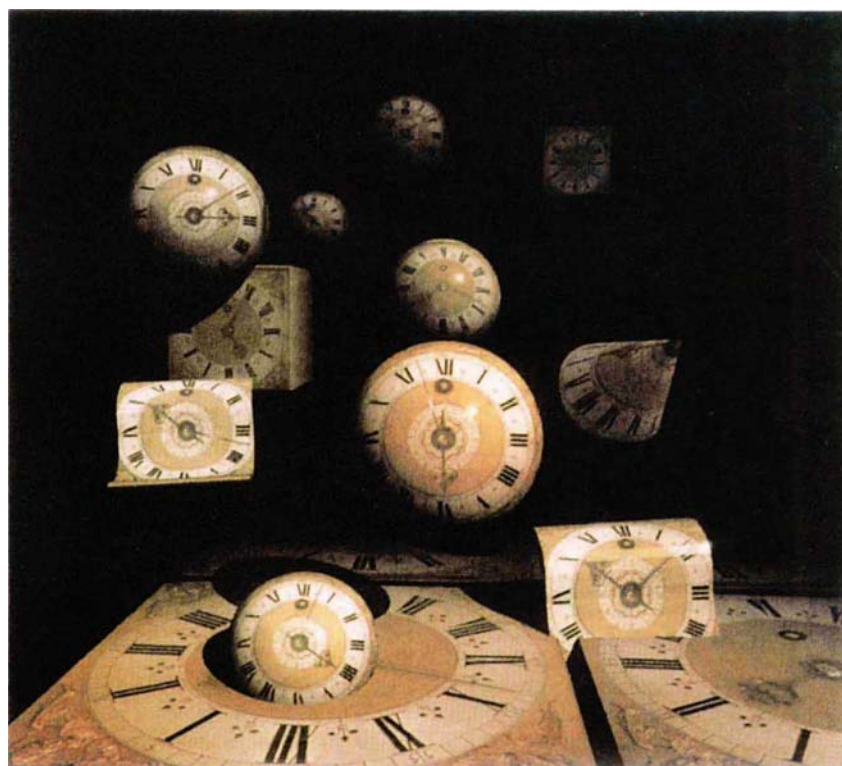
**A Theory for Everything.** By Jeremy Bernstein. *Copernicus* (Springer): 1996. Pp. 320. \$25, £18.50.

MOST scientists can't write, as any publisher will tell you. The few who can compose a literate, jargon-free essay are constantly in demand by the literati's house journals, always eager to point out that their tastes are so broad that they include practically everything, even science. This favoured coterie includes a good many life scientists, but the supply of physical scientists, particularly chemists, seems to be thinner.

Among the physicists who enjoy favour in US journalistic circles is Jeremy Bernstein. For some 30 years, he has been prolifically writing well-crafted scientific essays for journals as prestigious as *The New Yorker*, *The Atlantic Monthly* and *The New York Review of Books*. *A Theory for Everything* is an enjoyable collection of his factual and fictional pieces from these and other sources, some of them slightly retouched and some with a few editorial cuts restored.

By far the strongest contributions draw on Bernstein's reflections on the great scientists he has known since he was a young physicist in the 1940s. He never won a place in the front rank himself, as he is the first to admit, but he was talented enough to rub shoulders with the likes of Enrico Fermi ("a cold fish") and J. Robert Oppenheimer, who once recalled that he had read Proust as a young man — "in French, and by candlelight, while touring Corsica by bicycle".

As you might expect from someone with these connections, Bernstein is well aware of the overwhelming importance of the contributions made by a small number of exceptionally gifted minds. As he remarks,



"Time and space are now seen as dynamic quantities with each individual particle, or planet, having its own unique measure of time depending on where and how each is moving." From *The Illustrated A Brief History of Time*, an updated and expanded edition of Stephen Hawking's classic, which has sold more than 9 million copies and been translated into 30 languages. Bantam, \$37.50, £20.

perhaps a little nostalgically, "when people like [Julian] Schwinger and [Wolfgang] Pauli were at the height of their powers, giants walked the earth".

Schwinger, aptly nicknamed "His Majesty" by Pauli, is the subject of one of the best essays. Bernstein draws a light, well-balanced pen portrait of this solitary man, one of the architects of quantum electrodynamics, leavening the account with some amusing and unfamiliar anecdotes. He recalls that, at the end of one of Schwinger's talks on his attempts to reformulate quantum theory, the fearsomely critical Pauli remarked "Wasn't that rather trivial?", only to be silenced by Schwinger's reply: "I meant it to be".

One of Bernstein's prime virtues is that he maintains his readability without descending into facile abuse or idolization of his subjects. When he portrays Linus Pauling, for example, he gives appropriate weight to the brilliance of this great chemist, while not flinching from telling us of his eccentricities. Marie Curie is less well treated: in a review of Susan Quinn's biography of her, Bernstein follows the author in giving too much weight to Curie's private life, telling us far too little about how and why she became the century's pre-eminent woman scientist.

In writing of this type, intended to engage a wide audience in fundamental science, the author has sometimes to sail

close to the wind, compromising the standards of accuracy expected by pedants. But Bernstein is occasionally too lax, such as when he tells us that the X-rays first observed by Wilhelm Röntgen were emitted after he had excited a metal plate using his beam of electrons. In fact, as Röntgen knew, the radiation was emitted after the electrons had been slowed down when they struck the wall of his cathode-ray tube.

Factual accuracy is not a problem in the "lighter pieces" of fiction that end this collection. Bernstein introduces them by begging the reader's indulgence, as well he might. These stories are at best slight and at worst feeble — goodness knows how the famously selective *New Yorker* saw fit to publish some of them. To think that the journal accepted Bernstein's stories having rejected Saul Bellow's Nobel-cited *Seize the Day*...

It is a relief to hear that Bernstein has "more or less stopped writing fiction". No doubt he could make a good living by penning still more of his pleasing essays, but perhaps he might be persuaded to apply his considerable talents to something more substantial. He tells us that there is no biography of Schwinger: that would be a wonderful challenge for Bernstein and, if he were to rise to it, a treat for us. □

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# Fossil parks

Angela Milner

**Dinosaurs of the Flaming Cliffs.** By Michael Novacek. Anchor: 1996. Pp. 367. \$30.

**Dinosaurs of the East Coast.** By David Weishampel and Luther Young. Johns Hopkins University Press: 1996. Pp. 275. \$30.

**The Horned Dinosaurs: A Natural History.** By Peter Dodson. Princeton University Press: 1996. Pp. 323. \$35, £23.

HORNED dinosaurs, the east coast of North America and expeditions to Mongolia's Gobi Desert are the subjects of this trio of additions to the ever-growing library of dinosaur books aimed at the general reader. These three fall into the area between primary research literature and textbooks on the one hand, and the popular and glossy 'coffee-table' ware on the other. They are totally different in scope and subject matter, and are written by experts in the field. They cover topics in a depth that would otherwise be difficult for the nonspecialist to access.

The Gobi Desert is one of the two richest and most productive areas for dinosaur fossils in the world, the other being western North America. The spectacular natural treasures of the Gobi were brought to the attention of the world following the American Museum of Natural History's expeditions in the 1920s. Led by Roy Chapman Andrews, the expeditions' goal was to fulfil Henry Fairfield Osborne's hypothesis that our early ancestors lay waiting to be found in central Asia. Instead, they discovered several new dinosaurs and a remarkable series of nests and eggs attributed to the horned dinosaur *Protoceratops*.

The vertebrate palaeontologists now at the museum took up the challenge where their forebears left off, and in 1989 began a still continuing series of expeditions to the Gobi, organized jointly with the Mongolian Academy of Sciences. In *Dinosaurs of the Flaming Cliffs*, Michael Novacek, the expedition leader, tells a thoroughly absorbing adventure story, every bit as incident-filled as in Andrews' day, modern conveniences such as satellite navigation notwithstanding. He weaves together the dramas of day-to-day expedition life and the thrill of quite incredible fossil discoveries, with details of scientific methodology, interpretation of the fossil record and palaeoecology, to set the Gobi fauna in the context of Mesozoic evolution.

Some of the fruits of the team's endeavours have already captured the headlines: an *Oviraptor* preserved on its own nest of eggs, and an *Oviraptor* embryo. The embryo emerged from an egg type previously confidently assigned

to *Protoceratops*, disproving the identification and interpretation of *Oviraptor* as a *Protoceratops* nest-raider and egg-stealer — a belief that had held sway since the expeditions of the 1920s. Novacek discusses the treasures unearthed at the extraordinarily rich fossil site of Ukhaa Tolgod, including many exquisitely preserved skulls and skeletons of lizards and of mammals, which are likely to revolutionize our understanding of the history of marsupials and placentals. This eloquent and accessible blend of science and discovery should appeal to a wide readership, scientists and nonscientists alike, and may well inspire the next generation of palaeontologists.

Unlike the Gobi Desert, the east coast of North America is not readily associated with dinosaurs, although it yielded the New World's first discovery, *Hadrosaurus foulkii*, in 1858, some 16 years after Richard Owen erected Dinosauria based on finds in southern England. The long human association with the dinosaur-bearing beds of the eastern seaboard is chronicled by David Weishampel and Luther Young in *Dinosaurs of the East Coast*. The authors provide a meticulous, well-structured and thoroughly referenced account of 200 years of activities. They cover the collectors, the localities and the material, from many Triassic trackway sites to the challenging array of skeletal fragments and teeth from Jurassic and Cretaceous locations.

East coast dinosaurs are both rare and fragmentary, exposures are few and far between, and cliffs, cuttings and quarries often provide the only collection sites. But the area is as important to our understanding of evolution and palaeobiogeography as richer parts of the world. This book also serves as a field guide, complete with site maps, list of museum displays, sources of information on sites and advice on fossil collecting. Much information has been drawn together to create a comprehensive reference source for eastern North America.

The horned dinosaurs, Ceratopia, never reached eastern North America. The earliest members of the group inhabited Mongolia (as discovered by the Gobi expeditions in the 1920s) and dispersed in the Late Cretaceous period into western North America, which was isolated from the east by the Mid-Continental Sea. Their dispersal and evolution, culminating in *Triceratops*, is just one of the topics covered by Peter Dodson in *The Horned Dinosaurs*. A leading specialist on the ceratopians, he stamps authority on his comprehensive review of the past literature and the anatomy, phylogeny and biology of the group.

Dodson combines an easily understood anatomical primer with even-handed treatment of controversial issues, including the unending debate about dinosaur

extinction, and distinguishes clearly between facts and speculation. A liberal set of notes covers the text; the qualifiers, extra information and updates add a lot to the value of the whole. This book is ostensibly for the general reader, but also provides a ready up-to-date reference for students and specialists. □

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## New in paperback

**The Race Gallery: The Return of Racial Science** by Marek Kohn. Vintage, £6.99.

"A useful book... principally about current events rather than being a weighty discussion of underlying issues", Jonathan Marks, *Nature* **378**, 143 (1995).

**Ancestral Passions: The Leakey Family and the Quest for Humankind's Beginnings** by Virginia Morell. Simon and Schuster, \$16, £9.99. "Balanced and matter-of-fact", Leslie C. Aiello, *Nature* **377**, 111 (1995).

**Bugs in the System: Insects and Their Impact on Human Affairs** by May R. Berenbaum. Addison-Wesley, \$15. "The ultimate source of incredible entomological facts", S. J. Simpson, *Nature* **374**, 842 (1995).

**The Chosen Primate: Human Nature and Cultural Diversity** by Adam Kuper. Harvard University Press, \$15.95, £10.50. "An excellent introduction to an eternally awkward, though fascinating, area of anthropology", Mark Ridley, *Nature* **369**, 110 (1994).

**A View to a Death in the Morning: Hunting and Nature Through History** by Matt Cartmill. Harvard University Press, \$15.95, £10.50. "Elegant, erudite, stimulating", Adam Kuper, *Nature* **364**, 111 (1993).

**From Coastal Wilderness to Fruited Plain: A History of Environmental Change in Temperate North America from 1500 to the Present** by Gordon G. Whitney. CUP, £19.95. "Whitney's ecological history is as much about our global future as it is about our local past", Stuart L. Pimm, *Nature* **374**, 24 (1995).

**Green Imperialism: Colonial Expansion, Tropical Edens and the Origins of Environmentalism 1600-1860** by Richard H. Grove. CUP, £14.95, \$18.95. "A stimulating, if controversial, source for any ecologist or environmentalist", Quentin Cronk, *Nature* **376**, 652 (1995).

**The Day Before Yesterday: Five Million Years of Human History** by Colin Tudge. Pimlico, £9.99. "His knowledge of the evolutionary changes in the nonhominid components of the contemporary fauna is impressive", Bernard Wood, *Nature* **379**, 687 (1996).

# Endosome dynamics regulated by a Rho protein

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**Vesicular transport is a dynamic process that requires coordinated interactions between membrane and cytoskeleton. The mechanisms and molecules integrating these interactions are unclear. A Rho protein, RhoD, might provide a molecular link between membrane traffic and the cytoskeleton. Activated RhoD causes rearrangements of the actin cytoskeleton and cell surface, and governs early endosome motility and distribution.**

MEMBRANE transport is necessary for the biogenesis and maintenance of organelles and the transport of proteins and lipids through the secretory and endocytic pathways. Vesicular transport between organelles occurs in an environment whose shape, flexibility and organization is controlled by the cytoskeleton. Microtubules and actin filaments have been increasingly linked to the general eukaryotic transport apparatus. Actin is implicated in the development and maintenance of polarized growth in yeast<sup>1</sup>, and in endocytosis in yeast<sup>2</sup> and mammalian polarized epithelial cells<sup>3,4</sup>. Microtubules are involved in vesicular transport<sup>5</sup>, and are thought to control long-range bi-directional movement<sup>6</sup>.

Small GTPases are involved in many cellular processes<sup>7</sup>. Rab proteins regulate vesicular docking and fusion at various steps of biosynthetic and endocytic transport<sup>8-11</sup>, and GTPases of the Rho family regulate the actin cytoskeleton. Rho proteins transduce signals from plasma membrane receptors and control cell adhesion, motility and shape, as well as bud assembly and polarized growth in yeast and *Drosophila*<sup>12-17</sup>. Rho proteins are also thought to participate in membrane transport. RhoA regulates clathrin-independent endocytosis in *Xenopus* oocytes<sup>18</sup>, and activated mutants of RhoA and Rac1 impede the formation of clathrin-coated vesicles at the plasma membrane in mammalian cells<sup>19</sup>. However, the role of Rho proteins in organelle dynamics has not been explored.

Here we present the protein RhoD, which regulates early endosome dynamics and distribution, and causes rearrangements of the cell surface and actin cytoskeleton. We propose that RhoD is a good candidate for a molecule that coordinates membrane transport with the function of the cytoskeleton.

## Background

A cloning approach based on the polymerase chain reaction (PCR) indicated the existence of a new member of the Rho protein family<sup>20</sup>. To investigate its function we obtained a complementary DNA clone encoding the full-length protein and determined the nucleotide and deduced amino-acid sequence (Fig. 1a). Comparison of this sequence to the known members of the Rho family indicates a homology of 56–67%. We called this protein RhoD, because this analysis indicates that it is not an

isoform but rather a distinct member of the Rho family, more closely related to the RhoA, B, C group than to Rac and Cdc42. The protein is widely expressed in mouse tissues (Fig. 1b).

## Changes to the actin cytoskeleton

To investigate the function of RhoD, we transiently expressed in BHK cells the wild-type protein RhoD, RhoD<sup>G26V</sup> (a mutant deficient in GTP hydrolysis) and RhoD<sup>T31N</sup> (a mutant stabilized in the GDP-bound form), using the T7 vaccinia virus system<sup>21</sup>. Striking alterations of cell morphology were observed in cells expressing both RhoD and RhoD<sup>G26V</sup> (Fig. 2a–c). These cells show thin processes reaching lengths of 20–30 µm, some of which are firmly attached to the substratum, whereas others are motile. By time-lapse video microscopy we captured the growth of the extensions (see Supplementary Information), which are commonly observed (70%) among the transfected cells. F-actin was abundant in the structures (Fig. 2d), but microtubules were absent (data not shown). This effect is not restricted to BHK cells, as HeLa, NIH3T3, NRK and J774 cells exhibited the same phenotype. It is also not a result of the expression system used, as: (1) hydroxyurea was used to inhibit virus assembly, avoiding changes in the actin cytoskeleton<sup>21,22</sup>; (2) non-viral-mediated transient expression of RhoD gave similar results (data not shown); (3) expression of RhoD<sup>T31N</sup> induced minimal changes in cell morphology; and (4) activated mutants of Cdc42 and Rac1 similarly expressed did not exhibit the same effects as RhoD, but did cause the same phenotypic alterations on the actin cytoskeleton as reported<sup>15,16</sup> (data not shown).

The plasma membrane rearrangements induced by RhoD and RhoD<sup>G26V</sup> were accompanied by alterations of the actin cytoskeleton. In cells expressing wild-type or mutant RhoD (Fig. 2c), the actin stress fibres disappear from the cell body (Fig. 2d), although they remain intact in untransfected control cells (Fig. 2e), in cells expressing an unrelated protein (Fig. 2f, g) or in RhoD<sup>T31N</sup> (data not shown) under the same conditions. However, stress fibres were not stimulated by Rho<sup>V14</sup> using our expression system (not shown). As in the case of stress fibres, we found that focal adhesion-associated proteins, paxillin (Fig. 3), vinculin and phosphotyrosine-containing proteins (data not shown), disassemble from the cell periphery on expression of RhoD or RhoD<sup>G26V</sup> proteins. In contrast, the focal adhesions are still intact in cells expressing RhoD<sup>T31N</sup> (data not shown), Rac1<sup>V12</sup> and Cdc42<sup>V12</sup> (Fig. 3), as previously described<sup>16</sup>. We did not observe the presence of the above adhesion markers in the plasma-membrane processes formed by RhoD (Fig. 3a).

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## Regulation of early endosome dynamics

We next investigated the localization of RhoD in BHK cells overexpressing the myc-tagged protein. By a combination of confocal immunofluorescence and electron microscopy, RhoD was localized to the plasma membrane (Fig. 2*b*; this staining is not apparent following the extraction procedure for immunofluorescence) and vesicular structures throughout the cell (Fig. 4). Immunogold labelling also revealed an enrichment on putative endosomal compartments (data not shown). This localization was examined by confocal microscopy in BHK cells coexpressing the RhoD<sup>G26V</sup> protein with the human transferrin receptor. Fluorescein isothiocyanate (FITC)-labelled transferrin, internalized for 15 min, almost completely colocalized with RhoD, verifying that the labelled structures are indeed on the early endocytic/recycling pathway (Fig. 4) and perinuclear recycling compartment<sup>23,24</sup> (Fig. 4*b*, hash symbol). Moreover, overexpressed RhoD<sup>G26V</sup> altered the intracellular distribution and organization of early endosomes. First, early endosomes were more dispersed throughout the cell and extended into the RhoD-induced, actin-containing processes (Fig. 4*c–f*). Second, a striking alignment of early endosomes was often observed on expression of RhoD<sup>G26V</sup> (Fig. 4*b*). Third, the endosomes appeared less pleiomorphic and to a lesser extent connected by tubular processes than control cells. Electron-microscopy analysis indicated that the endosomes appear spherical, lacking the tubular extensions evident on control endosomes (Fig. 5).

To investigate whether RhoD could kinetically modify endocytic transport, we tested the effect of RhoD and RhoD<sup>G26V</sup> on the transferrin cycle *in vivo*. The number of surface transferrin receptors and the kinetics of internalization were unaltered. Transferrin recycling was decreased by approximately 10% (data not shown). Thus RhoD does not significantly affect the kinetics of transport from the plasma membrane to the early endosomes and along the recycling pathway. These data suggest that RhoD plays a different role in the dynamics of this compartment, such as regulating homotypic fusion, fission and/or motility. To allow the visualization of this process, we used the property of Rab5 which regulates early endosome homotypic fusion *in vitro*<sup>25</sup> and *in vivo*<sup>21</sup>. Expression of the GTPase-deficient mutant Rab5<sup>Q79L</sup> stimulates early endosome homotypic fusion and induces the appearance of enlarged endosomes<sup>26</sup> (Fig. 6*a, 7a*). To investigate the role of RhoD in endosome function we coexpressed Rab5<sup>Q79L</sup> with RhoD, RhoD<sup>G26V</sup> or RhoD<sup>T31N</sup>. When Rab5<sup>Q79L</sup> is expressed alone, large early endosomes are formed and accumulate around the nucleus<sup>26</sup>, and actin is recruited around the enlarged endosomes (Fig. 7*b*). When Rab5<sup>Q79L</sup> and wild-type RhoD (Fig. 6*a–c*) or RhoD<sup>T31N</sup> (data not shown) are coexpressed, the enlarged early endosomes are not altered in size or location. Actin is recruited around the Rab5-positive, large early endosomes (Fig. 6*c*), and RhoD is also localized to the endosomal membrane (Fig. 6*b*). In contrast, coexpression of RhoD<sup>G26V</sup> and Rab5<sup>Q79L</sup> abolishes the

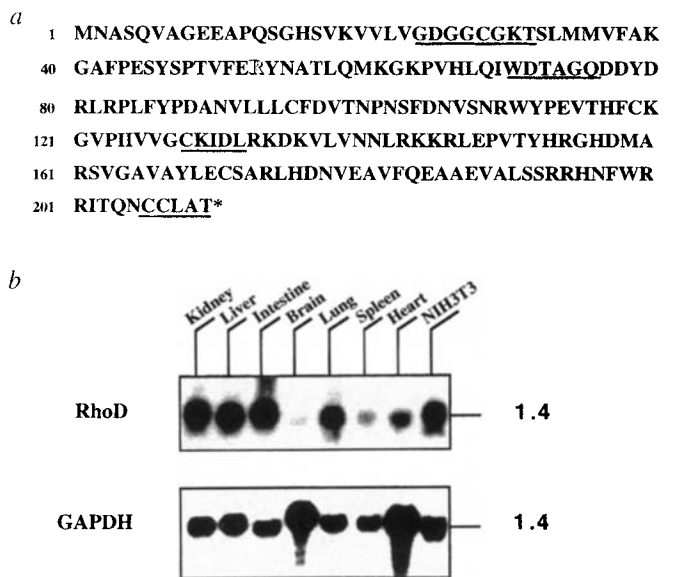
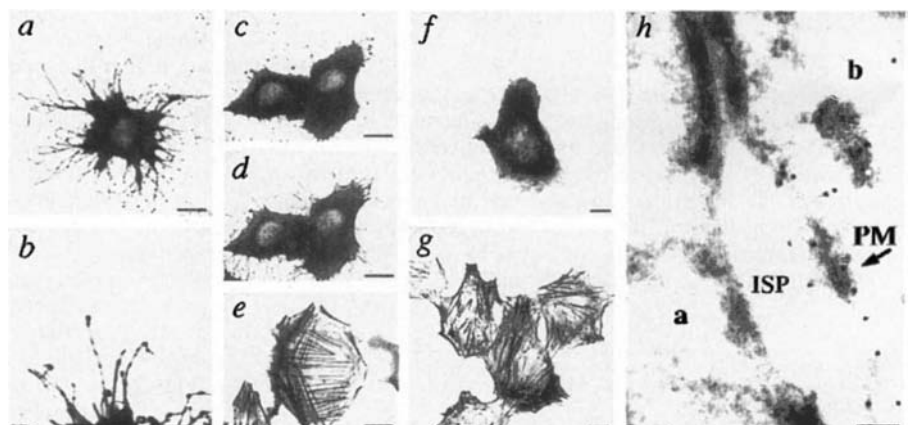


FIG. 1 Sequence and expression pattern of RhoD. *a*, Amino-acid sequence of mouse RhoD. Consensus motifs GXXXXGK[S,T] and DTAGQ are underlined. NKXDL, a conserved motif involved in GTP binding, is replaced in RhoD by CKIDL, underlined. The CAAX motif undergoing prenylation in RhoD is CCLAT, underlined. The asparagine residue in Rho proteins, the modification site for botulinum ADP-ribosyltransferase<sup>12,33</sup>, is replaced in RhoD by arginine, white letter. *b*, Northern blot analysis of total RNA from adult mouse organs hybridized with RhoD cDNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to control loading and RNA quality. RNA markers are indicated.

expansion of the endosomes, which are no longer perinuclear but appear scattered throughout the cell (Fig. 6*d–f*). Surprisingly, in view of the effect of RhoD and RhoD<sup>G26V</sup> on the disassembly of actin stress fibres, actin is still localized around the structures formed. Therefore the RhoD mutant protein neither prevents the recruitment of actin around, nor induces the disassembly of actin from, the endosomal membrane.

We performed two control experiments to exclude the possibility of a nonspecific effect of RhoD on the actin cytoskeleton. First, treatment of cells with 1  $\mu\text{g ml}^{-1}$  cytochalasin D, which causes F-actin depolymerization (Fig. 7*d*), had no effect on the formation of the endosomal structures or on their maintenance (Fig. 7*c*). Second, activated mutants of other members of the Rho protein family with established effects on the actin cytoskeleton, Rac1<sup>V12</sup> (Fig. 6*g*), Cdc42<sup>V12</sup> (Fig. 6*h*) or RhoA<sup>V14</sup> (Fig. 6*i*), did not impede the effects of Rab5<sup>Q79L</sup> on endosomal morphology. Thus

FIG. 2 RhoD induces plasma-membrane process formation, alters actin stress fibres, and localizes to the plasma membrane. *a*, BHK cell expressing myc-tagged RhoD<sup>G26V</sup> visualized by indirect immunofluorescence. *b*, Higher magnification of membrane processes. *c*, NRK cells expressing RhoD<sup>G26V</sup> labelled with an antibody recognizing myc-tagged RhoD<sup>G26V</sup> and *d*, rhodamine-labelled phalloidin. *e*, Stress fibres in a control cell. *f*, NRK cells expressing myc-Rab17 stained with rhodamine-labelled phalloidin. *g*, Immunoelectron microscopy on BHK cells expressing myc-tagged RhoD. *h*, Overview of a transfected (*b*) and a control cell (*a*). ISP, intercellular space; PM, plasma membrane. Scale bars: *a–g*, 10  $\mu\text{m}$ ; *h*, 100 nm.





the effect on early endosomes seen with RhoD<sup>G26V</sup> is highly specific.

### RhoD regulates endosome motility

To investigate the mechanism by which RhoD interferes with the formation of the large endosomes in response to Rab5<sup>Q79L</sup>, we monitored this process by time-lapse video microscopy. In cells expressing the Rab5<sup>Q79L</sup> mutant alone we observed rapid vesicle motility (see Supplementary Information). Endosome movement facilitated the contact between endocytic vesicles, causing progressive enlargement of endosomes by homotypic fusion at the cell periphery and movement towards the nucleus (see, for example, the movement and fusion of endosomes indicated by arrowheads in Fig. 8, panels 1–6). This analysis further indicated that endosomes become less motile as they grow. Homotypic fusion between endosomes seems to be asymmetrical, with one endosome expanding by assimilating the other (Fig. 8, panels 5, 6). In contrast, endosome motility was severely reduced in cells coexpressing Rab5<sup>Q79L</sup> and RhoD<sup>G26V</sup> (Fig. 8, panels 7, 8) (see Supplementary Information). Consistent with the immunofluorescence analysis (Fig. 4), the motionless endosomes were spread throughout the cells with extended processes. Occasionally, when contact occurred, endosomal fusion events were observed (see Supplementary Information), but it seems that the reduced endosomal motility caused by RhoD<sup>G26V</sup> might prevent the excessive fusion activity enhanced by Rab5<sup>Q79L</sup> alone. Cytosol containing overexpressed RhoD or RhoD<sup>G26V</sup> did not inhibit the homotypic fusion between early endosomes *in vitro* (data not shown), lending further support to the idea that RhoD does not act at the fusion stage. These results therefore suggest that the alterations of RhoD<sup>G26V</sup> on the endosome dynamics are primarily due to an inhibition of intracellular motility of this organelle.

### Discussion

Rho proteins share the property of regulating the actin cytoskeleton but they have different effects on actin organization. Expression of RhoA promotes assembly of stress fibres<sup>14</sup>, Rac1 generates lamellipodia<sup>15</sup>, and Cdc42 triggers actin polymerization and induces filopodia<sup>16</sup>. We have identified RhoD, a protein that is localized to the plasma membrane and early endosomes. When overexpressed, RhoD and RhoD<sup>G26V</sup> induce F-actin-containing membrane processes and the disassembly of stress fibres and focal adhesions from the cell periphery. The effects of RhoD are, therefore, in sharp contrast to those induced by the other Rho family members. Furthermore, RhoD plays an important role in endosome organization. RhoD<sup>G26V</sup> causes scattering of the endosomes throughout the cell and impairs Rab5<sup>Q79L</sup>-stimulated homotypic fusion.

We sought to identify the mechanism by which RhoD affects early endosome dynamics. Unlike Rho and Rac<sup>19</sup>, RhoD did not alter the kinetics of receptor-mediated endocytosis and recycling. Moreover, neither wild-type RhoD or RhoD<sup>G26V</sup> interferes with the homotypic fusion between early endosomes in a standard *in vitro* assay (data not shown), which depends on Rab5 activity<sup>25</sup> excluding a direct inhibitory effect on the fusion step. Time-

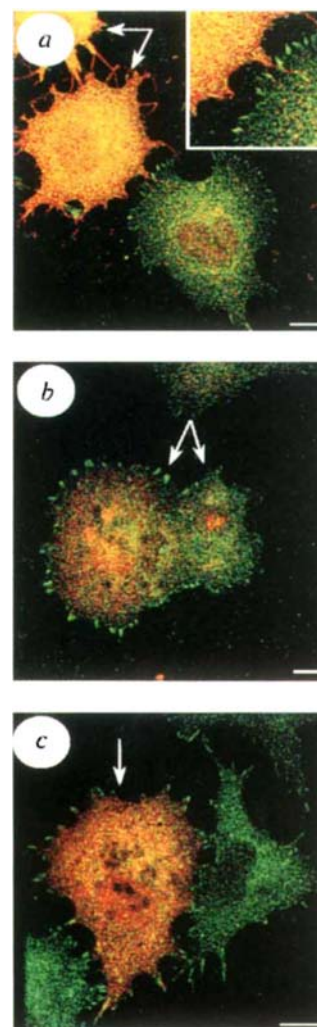


FIG. 3 Paxillin localization in BHK cells expressing RhoD<sup>G26V</sup>. BHK cells were either transfected with RhoD<sup>G26V</sup> (a), Rac1<sup>V12</sup> (b), or Cdc42<sup>V12</sup> (c). Double immunofluorescence was performed to detect RhoD (a), Rac1 (b) or Cdc42 (c) and paxillin. Only the overlays are shown here; RhoD, Rac1 and Cdc42 are visualized in red–orange colour, and paxillin in green. Arrows indicate the transfected cells. The insert in a is a higher magnification to demonstrate the loss of paxillin localization at the cell periphery in the cell expressing RhoD<sup>G26V</sup>. Scale bars, 10  $\mu$ m.

lapse video microscopy analysis suggested a different explanation: the RhoD mutant seems to exert its effect by inhibiting organelle motility. This does not necessarily imply that RhoD is an inhibitor of endosome dynamics, but rather that its normal GTP/GDP cycling (perturbed in the mutant) regulates this activity. In a dynamic equilibrium between fusion and fission, the RhoD

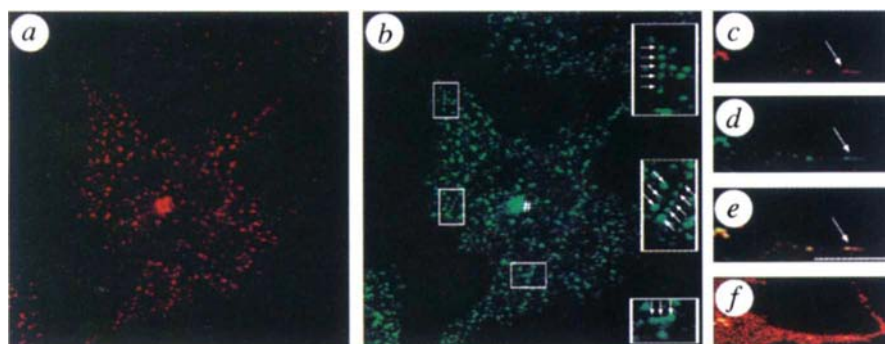


FIG. 4 Localization of RhoD and internalized FITC-labelled transferrin, which was internalized for 15 min at 37 °C in BHK cells expressing RhoD<sup>G26V</sup> and the human transferrin receptor. a, RhoD<sup>G26V</sup> staining, and b, FITC-labelled transferrin. In b, arrows indicate aligned endosomes with inserts showing magnifications of these areas; the hash sign indicates the perinuclear recycling endosome. c–f, Membrane processes formed upon expression of RhoD<sup>G26V</sup>. c, RhoD<sup>G26V</sup> staining; d, FITC-labelled transferrin; and e, the overlay of c and d. f, This image has a pseudocolour to demonstrate the shape of the membrane process. Scale bar, 10  $\mu$ m.

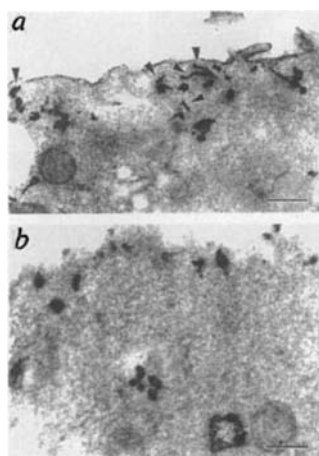


FIG. 5 Morphological alteration of early endosomes in cells expressing RhoD<sup>G26V</sup>. *a*, Control BHK cells showing early endosomes with several tubular connections (arrowheads). *b*, In BHK cells expressing RhoD<sup>V26G</sup>, endosomes are spherical and lack tubular connections. Scale bars, 250 nm.

mutant might shift the balance towards fission, dispersing the endosomes and decreasing the tubular connections (Figs 4 and 5). Endosome motility might be important, for example in the case of cell migration, where membrane dynamics are critical and adhesion molecules and surface receptors are internalized at the rear of the cell and recycled to the leading edge (see refs 27–29 for reviews).

It is likely that RhoD function depends on the cytoskeleton,

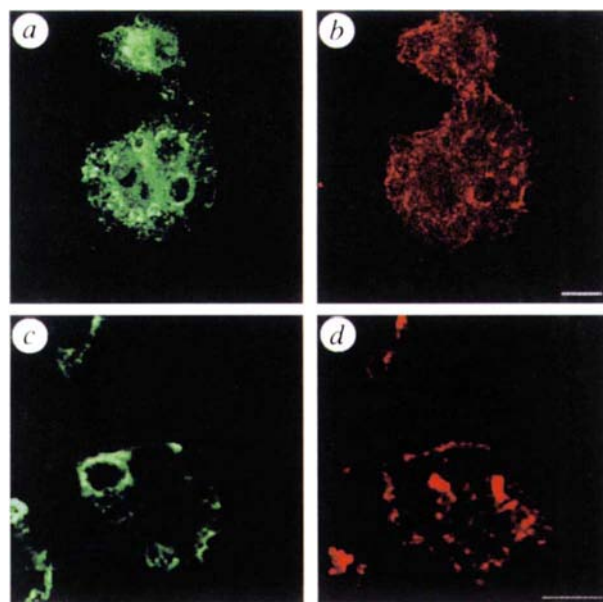


FIG. 7 Effect of cytochalasin D on the formation and maintenance of the enlarged endosomes formed by Rab5a<sup>Q79L</sup>. BHK cells were transfected with a plasmid encoding Rab5a<sup>Q79L</sup>. 30 min after transfection, 1 μg ml<sup>-1</sup> cytochalasin D was added to half of the samples, and the others were allowed to express the protein in the absence of the drug as a control. The cells were fixed and processed for immunofluorescence 4 h after transfection. In control cells, *a*, Rab5 staining, and *b*, the F-actin. In cells treated with cytochalasin D, *c*, Rab5 staining, and *d*, the F-actin. Scale bars, 10 μm.

FIG. 6 Effect of RhoD and RhoD<sup>G26V</sup> on early endosome enlargement induced by Rab5a<sup>Q79L</sup>. BHK cells were co-transfected with Rab5a<sup>Q79L</sup> and either RhoD (*a–c*) or RhoD<sup>G26V</sup> (*d–f*). Immunofluorescence staining of Rab5a (*a, d*), RhoD (*b, e*), and rhodamine phalloidin to visualize F-actin (*c–f*). BHK cells were co-transfected with Rab5a<sup>Q79L</sup> and Rac1<sup>V12</sup> (*g*), Cdc42<sup>V12</sup> (*h*) or RhoA<sup>V14</sup> (*i*). Only the Rab5a staining is shown. Scale bar, 10 μm.

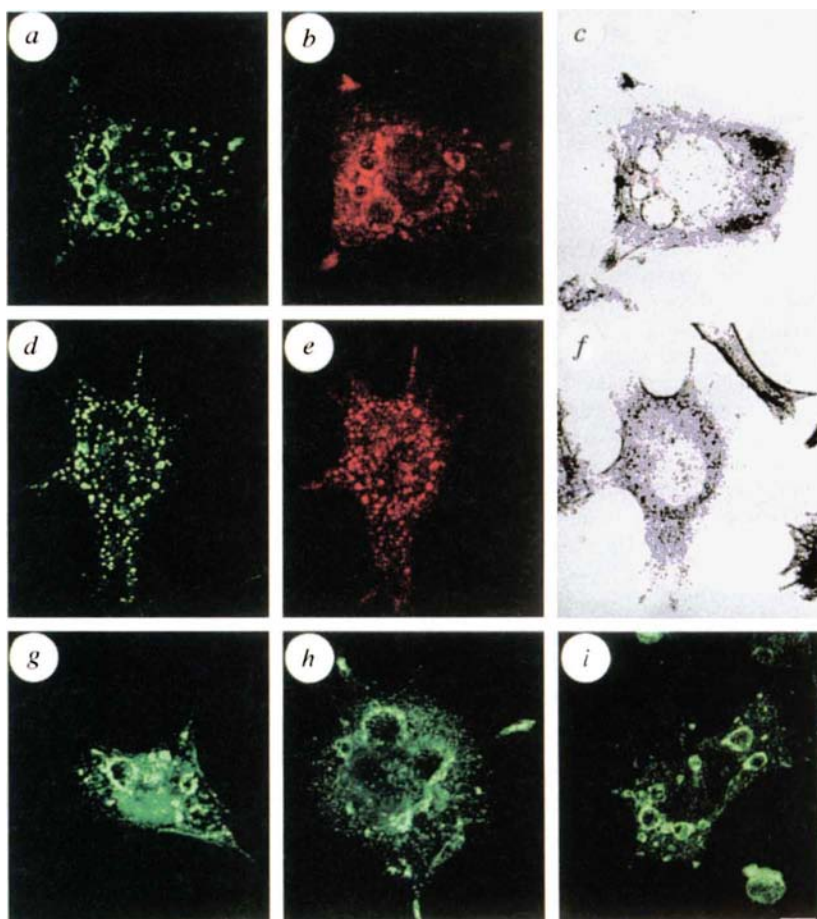
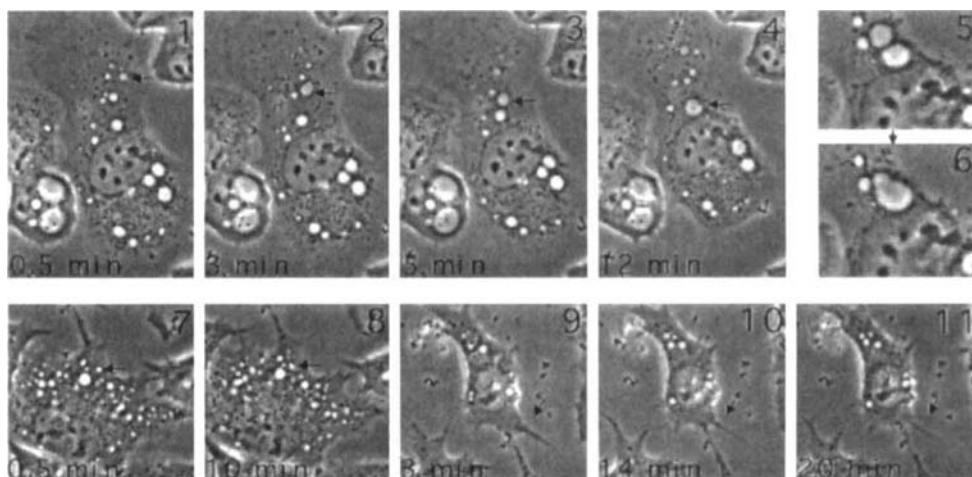


FIG. 8 Time-lapse video microscopy of BHK cells expressing Rab5a<sup>Q79L</sup> (panels 1–6) or coexpressing Rab5a<sup>Q79L</sup> and RhoD<sup>G26V</sup> (panels 7–11) (see Supplementary Information). Panels 5 and 6 show asymmetric endosome fusion. Panels 9–11 show membrane process extension in a cell coexpressing Rab5a<sup>Q79L</sup> and RhoD<sup>G26V</sup>. Panels 1–4 are 62  $\mu\text{m} \times 89 \mu\text{m}$ ; panels 7–11 are 63  $\mu\text{m} \times 63 \mu\text{m}$ .



given its effect on actin organization, and the observation that the endosomes are often aligned suggests movement along cytoskeletal filaments. Despite the lack of effect of cytochalasin D on endosome expansion (Fig. 7), actin remains one obvious candidate. Indeed, although this drug disrupts the supramolecular organization of the actin network, it is not clear whether all filaments are affected equally<sup>30</sup>. In this respect, it is noteworthy that, under conditions where actin stress fibres disassemble upon RhoD expression, actin remains associated with the endosomal membrane. The discovery of unconventional myosins containing GAP domains for Rho proteins<sup>31,32</sup> also suggests that actin may be involved. However, the movement of the endosomes towards the perinuclear region suggests that microtubules also participate, and it is conceivable that both types of filaments could be sequentially involved. Our findings showing that RhoD exerts a regulatory role on the actin cytoskeleton and endosome dynamics provide direction for further studies intended to unravel the mechanisms governing the interactions between membrane and cytoskeleton. □

## Methods

**Cloning of rhoD, generation of expression constructs, and northern blot analysis.** In a PCR screen to identify new GTP-binding proteins a PCR fragment encoding a Rho-related protein was isolated<sup>20</sup>. Using a combination of 5' and 3' rapid amplification of cDNA ends (RACE)<sup>34</sup>, the entire coding region was isolated from mouse kidney RNA. After cDNA synthesis using oligo dT, the 5' end was tailed with poly(G) residues and PCR was performed using primer 5'-CAGGTTTACCCCTTCATCTG-3' specific for RhoD, and an upstream poly(C) primer. A second round of PCR used a nested antisense RhoD-specific primer, 5'-TCGAGGATC-CAGAGTGGCATTATAGCGC-3' and the upstream poly(C) primer. To extend the 3' end, a first round of PCR was carried out using an upstream sense primer in RhoD 5'-CCTTCCCAGAGAGCTACAG-3' and a 3' oligo dT primer. A second round of PCR used a nested sense primer in the RhoD sequence 5'-GCCGAATTCGGATCCAGAGTACAGTCCACAGTGTGGA-3'. RhoD cDNA was cloned in frame into the pGEM 1 vector containing the c-myc tag (pGEM-myc) at the 5' end. The G26V mutant of RhoD was constructed by PCR using the oligonucleotide 5'-GGTGGGCGACGTGGGCTGCGGG-3', according to ref. 35 and cloned into pGEM-myc. Rac1<sup>V12</sup> and RhoA<sup>V14</sup> were subcloned into pGEM-myc. Cdc42<sup>V12</sup> was constructed and cloned into pGEM-myc for expression. For northern blot analysis, total RNA was isolated from adult mouse organs according to standard procedures. Total RNA (25  $\mu\text{g}$ ) was loaded on a formaldehyde gel, transferred to Genescreen plus membrane and probed with a PCR fragment corresponding to the 3' end of the cDNA.

**Immunofluorescence.** BHK cells were transiently transfected, fixed and immunofluorescence performed essentially as described<sup>21</sup>. To detect the myc-tagged proteins, the 9E10 monoclonal antibody (ATCC19) was used. To visualize the actin stress fibres, rhodamine-labelled phalloidin (Molecular Probes) was added with the secondary antibodies (Dianova). Staining with Rab5a polyclonal antibody was as described<sup>36</sup>. Polyclonal rabbit antiserum was raised against histidine-tagged RhoD expressed in *Escherichia coli*. In the experiment of Fig. 3, rabbit polyclonal antibodies against RhoD, Rac1 (Santa Cruz, used at 1:50 dilution) or Cdc42 (as described in ref. 35 and used at 1:50 dilution) and a

monoclonal antibody recognizing paxillin (Zymed, used at 1:50 dilution) were used. Cy5-labelled anti-mouse secondary antibody was a gift from S. Reinsch and was used at 1:50 dilution. Samples were visualized using the EMBL confocal scanning laser-beam microscope<sup>37</sup>. The 529, 476 and 633 laser lines of the Argon-ion laser were used for excitation of rhodamine, fluorescein and cy5-labelled samples, respectively. Images were recorded and imported into either Adobe Photoshop or NIH Image graphic programs for compilation.

**Electron microscopy.** For immunoelectron microscopy, BHK cells were transfected as described<sup>21</sup>, fixed in 8% paraformaldehyde in 250 mM HEPES, pH 7.35, scraped from the culture dish, pelleted and processed for cryosectioning<sup>38</sup>. Thawed sections were labelled by sequential incubations with the 9E10 culture supernatant, followed by secondary antibody and 10-nm protein A-gold. To compare the morphology of the endosomes after expression of RhoD<sup>G26V</sup>, we co-transfected this DNA and human transferrin receptor. After 4 h, cells were washed and incubated continuously for 5 min with 25  $\mu\text{g ml}^{-1}$  human transferrin conjugated to peroxidase. At this concentration only those cells expressing a high level of transferrin receptor are labelled.

**FITC transferrin uptake.** BHK cells were infected as described<sup>21</sup>, and co-transfected with plasmids encoding RhoD<sup>G26V</sup> and the human transferrin receptor. 4 h after transfection (the medium was devoid of serum), the coverslips were incubated in a humidified chamber with 50  $\mu\text{g ml}^{-1}$  FITC-labelled transferrin for 15 min at 37 °C. Coverslips were then rinsed in PBS and fixed in 3% PFA, permeabilized in 0.1% triton, and the 9E10 monoclonal antibody recognizing the myc-tagged RhoD was added, followed by rhodamine-labelled donkey anti-mouse IgG.

**Time-lapse video microscopy.** BHK cells were transfected as above, and 2 h later cyclohexamide was added for 1 h (ref. 21). Cells were washed 8 times for 1 min to remove the cyclohexamide, and video microscopy was carried out using a Zeiss Axiocvert 10 inverted microscope equipped with a cooled slow-scan CCD (charge-coupled device) camera (Photometrics CH250) connected to a SUN workstation. Images were acquired every 30 s at 37 °C with low light exposure, at  $\times 63$  magnification (Zeiss Plan-Apochromat lens, 63  $\times$  1.4). A series of 30–100 images were taken, corresponding to 15–50 min of observation. The images were processed and animated using the software package KHOROS and NIH-Image.

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# Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction

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**The human cytomegalovirus genome encodes proteins that trigger destruction of newly synthesized major histocompatibility complex (MHC) class I molecules. The human cytomegalovirus gene *US2* specifies a product capable of dislocating MHC class I molecules from the endoplasmic reticulum to the cytosol and delivering them to the proteasome. This process involves the Sec61 complex, in what appears to be a reversal of the reaction by which it translocates nascent chains into the endoplasmic reticulum.**

CYTOLYTIC T cells eradicate virus-infected cells by recognition of virus-derived peptides in a physical complex with MHC class I molecules<sup>1</sup>. In this way, the cell advertises to the immune system that it harbours a pathogen, and invites its destruction.

The selective pressure exerted by the immune system on viruses has led them to acquire, in the course of their evolution, a set of remarkable strategies with which they can elude cytolytic T cells. Certain viruses inhibit surface expression of class I–peptide complexes, thereby preventing destruction of the host cell in which they replicate<sup>2</sup>. In human cytomegalovirus (HCMV)-infected cells, this mechanism involves the rapid degradation of MHC class I molecules<sup>3,4</sup>, a process in which the HCMV genes *US2* and *US11* play a key role<sup>4,5</sup>. Such a multiplicity of mechanisms to cripple MHC class I expression suggests that cytolytic T cells are pivotal in the host defence against HCMV.

MHC class I molecules consist of two subunits, a heavy chain and  $\beta_2$ -microglobulin, whose association is required for peptide binding in the ER and for efficient transport of the complex out of the ER<sup>6</sup>. The heavy chain is targeted to the endoplasmic reticulum (ER) membrane by an amino-terminal, cleaved signal sequence, contains a glycosylated luminal domain, and has a carboxy-terminal membrane anchor (type I membrane protein). The light chain  $\beta_2$ -microglobulin is a secreted protein with a cleavable signal sequence. Both proteins are translocated across the ER membrane during their synthesis by an apparatus whose major constituent is the Sec61 complex. This membrane protein complex consists of three subunits,  $\alpha$ ,  $\beta$ ,  $\gamma$ , and probably forms a protein-conducting channel<sup>7</sup>.

In cells expressing the HCMV *US11* gene product, a type I membrane protein, the heavy-chain molecules are inserted into the ER membrane and are glycosylated cotranslationally, but shortly thereafter they are rapidly transported back into the cytosol, where they are deglycosylated by an *N*-glycanase and degraded by the proteasome<sup>5</sup>. How dislocation of the polypeptide chains occurs is unknown. However, a similar process may also occur in normal cells and may correspond to the process referred to as ER degradation<sup>8,9</sup>. Several membrane proteins that misfold in the ER are degraded following the attachment of ubiquitin<sup>10–13</sup>. Involvement of the ubiquitin pathway<sup>14</sup>, and inhibition of breakdown by agents that target the proteasome<sup>15</sup> imply that there is a cytosolic component to such degradation. Again, the mechanism by which these proteins are extracted from the membrane and transferred to the proteasome is unknown.

We have now found a second HCMV gene product, encoded by *US2*, that also triggers dislocation of newly synthesized class I heavy chain molecules into the cytosol for degradation by the proteasome. Characterization of the fate of MHC class I molecules in cells expressing the *US2* gene product illuminates the underlying molecular mechanisms. *US2* binds to newly synthesized class I molecules and escorts them into the cytosolic compartment where the heavy chains, and possibly *US2*, are deglycosylated by a *N*-glycanase. The deglycosylated breakdown intermediate is associated with the Sec61 complex, suggesting that retrograde transport occurs through the same protein-conducting channel that allowed the original membrane insertion of the heavy chain. The deglycosylated intermediate is also associated with the

proteasome, in further support of the idea that this cytosolic organelle is the site of its subsequent degradation. When misfolding of class I molecules is induced in cells that do not express US2, the proportion of heavy chains found in association with the Sec61 complex increases dramatically immediately before their destruction. We therefore propose that degradation of misfolded proteins in the ER may generally occur by the retrograde transport of polypeptides through the protein-conducting channel, followed by their degradation in the cytosol by the proteasome.

### Breakdown of MHC class I molecules

In U373 cells transfected with *US2* (*US2*<sup>+</sup> cells), MHC class I molecules are markedly less stable than in control cells. In a pulse-chase experiment, most of the newly synthesized class I heavy chains (HC) are degraded within 30 min, regardless of whether they are unassembled—as detected by immunoprecipitation with the antibody RaHC—or properly folded and associated with  $\beta_2$ -microglobulin as detected by the conformation-sensitive monoclonal antibody W6/32 (ref. 16) (Fig. 1a, b). In the presence of the proteasome inhibitors lactacystin<sup>17</sup>, carboxybenzyl-leucyl-leucyl-leucinal (ZL<sub>3</sub>H)<sup>5</sup> or carboxybenzyl-leucyl-leucyl-leucyl-vinylsulphone (ZL<sub>3</sub>VS; M. B., J. McMaster and H. L. P., unpublished observations), degradation is inhibited, but not completely blocked, and a 40K breakdown intermediate is observed (Fig. 1a). The intermediate, which comigrates with class I HC from tunicamycin-treated cells, is the product of an *N*-glycanase-catalysed reaction, as demonstrated by analysis of the intermediate by isoelectric focusing (data not shown, and ref. 5). Other intermediates, such as ubiquitin-conjugated species, could not be detected.

We next did experiments to confirm that even properly folded MHC class I molecules can be degraded in a US2-dependent fashion. The assembly of class I molecules requires an oxidizing environment in the ER<sup>18</sup>. In cells that do not express US2, free heavy chains are converted into properly folded class I molecules, the W6/32 reactive complex<sup>19,20</sup> (Fig. 2a). In the presence of the reducing agent dithiothreitol (DTT), there is a stark reduction in the quantity of properly folded, W6/32 reactive class I molecules. Instead, we found free heavy chains that tend to form aggregates. After 40 min of chase, these chains abruptly disappeared. Removal of DTT allows efficient refolding<sup>21</sup> of MHC class I molecules (Fig. 2). When cells are first incubated with DTT and the reducing agent is then removed, free class I HC disappear, and efficient refolding of MHC class I molecules occurs.

In *US2*<sup>+</sup> cells, upon DTT removal, the decrease of free heavy chains coincides with a transient increase of W6/32 reactive, folded heavy chains (Fig. 2b). Therefore, these ER-resident glycosylated free heavy chains are first folded and subsequently degraded in a US2-dependent manner.

To link a precursor-product relationship of the MHC class I HC precursor and the deglycosylated intermediate with intracellular location we did a pulse-chase experiment in the presence of the proteasome inhibitor ZL<sub>3</sub>H in combination with subcellular fractionation (Fig. 2c). The 1000g pellet, comprising debris, cell remnants and nuclei, together with trapped cytosol, reveals the presence of both precursor and product. When the postnuclear supernatant fraction of the 1000g sedimentation is further analysed, most of the deglycosylated class I breakdown intermediate is cytosolic (100,000g supernatant), in agreement with the suggestion that it would normally be broken down by the proteasome (see below). Some of the deglycosylated breakdown intermediate is present in the 100,000g pellet, where it reaches a maximum at 10 min of chase, after which it remains constant. This may represent material associated with a larger cytosolic complex such as the 26S proteasome. In the cytosol, some glycosylated precursor is present at equal intensities at the 1- and 5-min chase points but not later, suggesting that at least some of the deglycosylation reaction may also occur to class I HC released in the cytosol. In the 10,000g pellet which contains microsomes, the intact heavy chain essentially showed the same time course as the total cellular population. Taken together, these results demonstrate that US2 triggers the

dislocation of glycosylated class I HC into the cytosol where they are deglycosylated by a *N*-glycanase and subsequently degraded by the proteasome.

### ATP-dependent dislocation

As a first step towards the clarification of the mechanism of dislocation, we tested whether the process is ATP dependent. *US2*<sup>+</sup> cells were depleted of ATP by addition of 2-deoxyglucose and antimycin A<sup>22</sup> immediately after a 1-min pulse-labelling period. Conversion of the class I HC precursor to the deglycosylated product is significantly inhibited (Fig. 3a). The fraction of class I HC that fails to undergo conversion to the deglycosylated product is accounted for by an increase in folded, W6/32 reactive class I molecules, indicating again that degradation and folding are competing processes.

The ATP dependence of dislocation was confirmed in an experiment in which ATP depletion was imposed before, at the onset of, or at later times in the chase period (Fig. 3b). Within 1 min of ATP depletion, protein synthesis is reduced by more than 90%. ATP depletion produced a marked reduction in the relative amount of dislocated class I HC. This was again accompanied by an increase in the relative amount of W6/32 reactive class I molecules. Protein folding in the ER can therefore continue, even at intracellular ATP levels where protein synthesis has essentially stopped. We conclude that the dislocation reaction requires ATP, like other transport processes across membranes.

### US2 association

Next, we addressed the role of US2. The US2 protein is probably a membrane protein containing a C-terminal transmembrane segment, which contains two of the three possible *N*-linked glycan attachment sites, whereas the third site is located N-terminal of the transmembrane segment. The *US2* gene product occurs in at

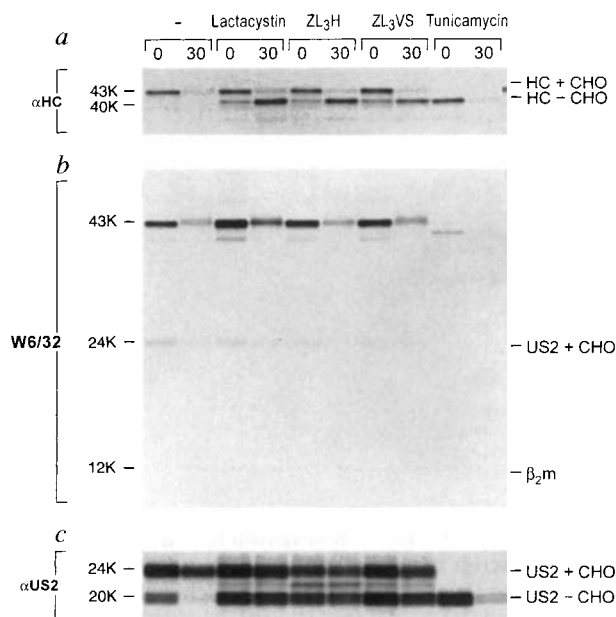


FIG. 1 US2 targets MHC class I molecules for proteasomal destruction. a, In *US2*<sup>+</sup> cells, MHC class I heavy chains (43K, HC + CHO) are rapidly destroyed and give rise to a deglycosylated breakdown intermediate (40K, HC - CHO) in the presence of proteasome inhibitors. CHO designates the *N*-linked oligosaccharide. b, Properly folded MHC class I molecules (W6/32 reactive material) form a complex with US2 (24K) and are targeted for destruction in *US2*<sup>+</sup> cells. The deglycosylated class I heavy chain cannot be detected in the W6/32 immunoprecipitates. The positions of migration of  $\beta_2$ m (12K) is indicated. c, US2 occurs in a glycosylated (24K, US2 + CHO) and a non-glycosylated form (21K, US2 - CHO). The non-glycosylated form of US2 is rapidly destroyed in a manner sensitive to proteasome inhibitors.

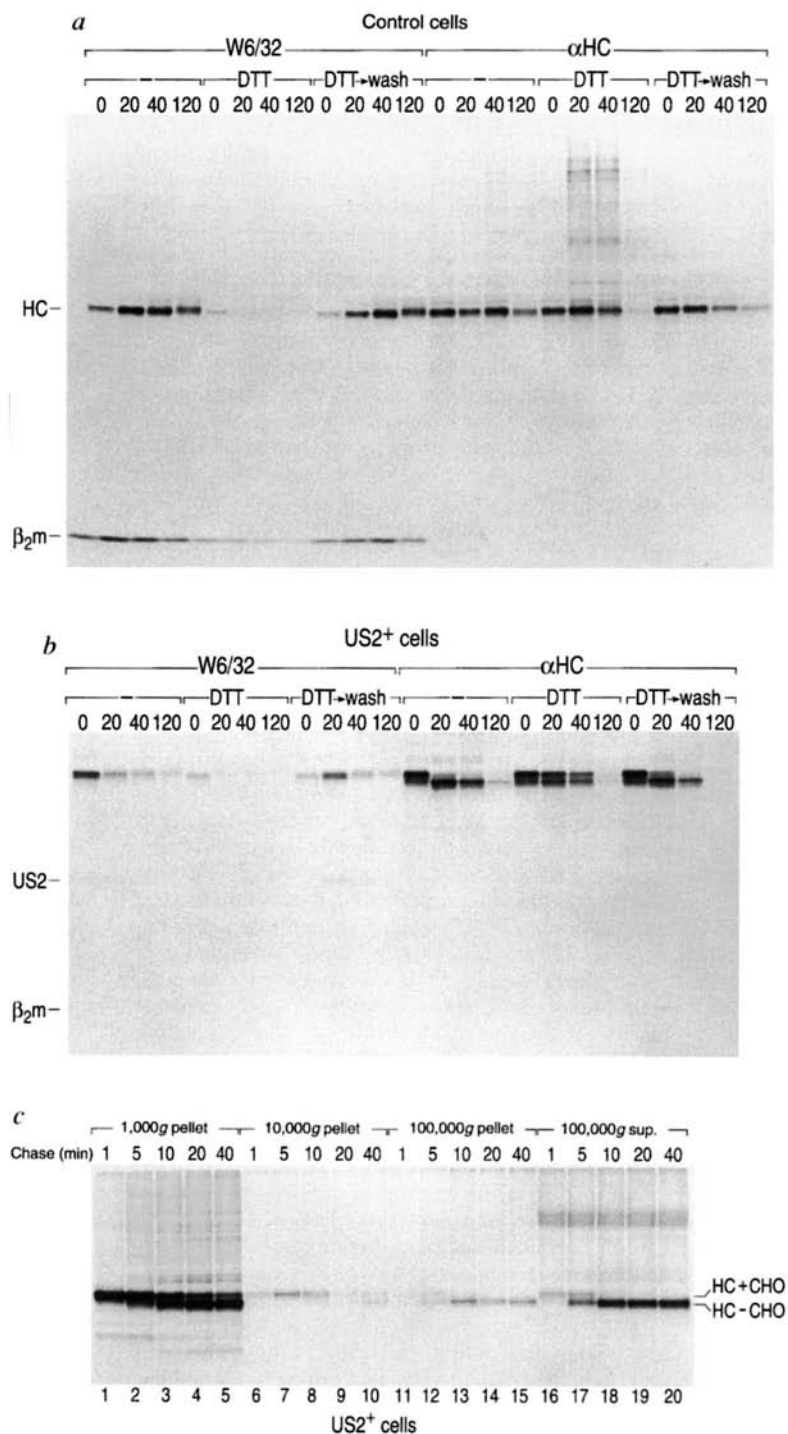


FIG. 2 Folded class I molecules are degraded in US2<sup>+</sup> cells and the deglycosylated intermediate is found in the cytosol. **a**, Class I molecules were recovered with either rabbit anti-heavy chain serum ( $\alpha$ HC) or W6/32 from control cells, cells treated with dithiothreitol (DTT), and cells treated with DTT followed by DTT wash-out, as indicated. All pulse-chase experiments were done in the presence of ZL<sub>3</sub>H. **b**, As **a**, but for US2<sup>+</sup> cells. Note the refolding of class I HC, as is evident from acquisition of W6/32 reactivity, upon removal of DTT. Note also the presence of glycosylated US2 in association with the refolded molecules. **c**, Deglycosylated breakdown intermediate (HC-CHO) occurs in the cytosol (100,000g supernatant), as assessed by a pulse-chase experiment combined with subcellular fractionation. Note that some of the deglycosylated breakdown intermediate can be sedimented after 1 h at 100,000g at later chase times. This fraction probably corresponds to proteasome-associated class I HC. CHO designates the *N*-linked oligosaccharide.

least two principal forms, both in virus-infected cells (T.R.J. and L. Sun, manuscript submitted) and in US2<sup>+</sup> cells (Figs 1c, 4a, b). These differ by the presence of a single high-mannose *N*-linked glycan, as seen from partial (not shown) and complete digestions of immunoprecipitated US2 with endoglycosidaseH (EndoH) (Fig. 4a), and from labelling experiments in the presence of tunicamycin (Fig. 1c). The occurrence of *N*-linked glycosylation indicates initial exposure of US2 to the lumen of the ER, and failure to acquire EndoH resistance suggests that it is an ER-resident protein, consistent with its activity on newly synthesized class I molecules. In a subcellular fractionation experiment, glycosylated US2 was indeed mostly found in a particulate fraction, whereas non-glycosylated US2 was located in the cytosol (data not shown). In pulse-labelling experiments even as short as 45 s, both forms are present (Fig. 4b, right). The non-glycosylated

form is readily degraded, but the glycosylated form is more stable (Fig. 1c). Degradation of the non-glycosylated form can be largely prevented by the proteasome inhibitors tested (Fig. 1c). Poor solubility in urea-containing isoelectric focusing (IEF) sample buffer precludes analysis by IEF of US2. Hence we cannot be certain that non-glycosylated cytosolic US2 originates from the action of an *N*-glycanase, but otherwise its fate is strikingly similar to that of the deglycosylated class I HC intermediate.

To test whether US2 acts directly on MHC class I HC, co-immunoprecipitation experiments were carried out. Lysates of labelled US2<sup>+</sup> cells were produced in digitonin, a relatively mild detergent, and immunoprecipitated with the antibodies indicated (Fig. 4a). After dissociation of immune complexes in SDS and dilution into NP-40-containing buffer, the proteins were reprecipitated with anti-US2 antibodies. Association of US2 with class I



molecules was observed. The glycosylated form of US2 was found predominantly in a complex with the folded, W6/32 reactive class I molecules (Figs 1, 2b), whereas non-glycosylated US2 was recovered mainly from anti-heavy-chain precipitates, which contain mostly class I breakdown intermediate. A similar reimmunoprecipitation in a pulse-chase experiment (Fig. 4b, right) confirms the US2-class I association. Because the amount of US2-complexed HC breakdown intermediate reaches a plateau at a time where the total amount of HC is already in steep decline we suggest that US2 escorts class I molecules into the cytosol. As expected, given its lectin-like nature<sup>23</sup>, calnexin exclusively interacts with the glycosylated form of US2 (Fig. 4a). Combined, our results demonstrate a direct interaction of US2 and class I molecules, both of which are delivered from the ER to the cytosol where they are destroyed. We have not detected similar complexes for US11 and class I HC (not shown).

### Association with the proteasome

Next, we tested whether a physical complex between class I heavy chains and the proteasome could be detected in inhibitor treated cells. Proteasome antibodies precipitate a number of polypeptides, including the characteristic  $\alpha$ - and  $\beta$ -subunits of relative molecular mass  $\sim 30$ K (Fig. 4b; confirmed by site-specific labelling of  $\beta$ -subunits with a radioactive peptide-vinylsulphone (M.B. and H.L.P., manuscript in preparation)). Reimmunoprecipitation with anti-heavy chain antibodies reveals the presence of the breakdown intermediate, but not of the glycosylated precursor, with maximum recovery at 7 min of chase (Fig. 4b). At this time, US2<sup>+</sup> cells contain both the precursor and the deglycosylated product, but only the latter associates with the proteasome. This association reaches a maximum before the total deglycosylated population peaks (20 min). Complexes between MHC class I heavy chains and the proteasome can also be detected in US11-expressing cells but not in control cells, even though the latter always contain some free class I HC (Fig. 4c). Taken together, these results support the notion that the proteasome is the site of further degradation of the deglycosylated heavy chains.

### Association with the Sec61 complex

Given the rapidity of the dislocation and breakdown reaction, newly synthesized class I heavy chains are unlikely to have strayed far from their point of insertion, the Sec61 complex. We therefore explored the possible involvement of the translocation complex in the dislocation reaction by co-immunoprecipitation. Digitonin extracts were prepared from US2<sup>+</sup> cells treated with the proteasome inhibitor ZL<sub>3</sub>H and immunoprecipitated with Sec61 $\beta$  antibodies. These conditions leave the Sec61 complex intact<sup>24</sup>. The immune complexes were dissociated in SDS and reimmunoprecipitated with RaHC. Only the deglycosylated class I heavy chains were found to be associated with Sec61 in US2<sup>+</sup> cells (Fig. 5a). A similar complex of the deglycosylated heavy chain and the Sec61 complex can also be recovered with antibodies against the  $\gamma$ -subunit of the Sec61 complex (our unpublished observations). In the absence of ZL<sub>3</sub>H, no complexes of class I HC and Sec61 were detectable (not shown). The amount of class I breakdown intermediate that is associated with Sec61 increases with time and reaches its maximum when the breakdown intermediate is actually destroyed. Because only deglycosylated class I HC are complexed with Sec61, the reportedly cytosolic *N*-glycanase<sup>25</sup> must be able to act on the class I heavy chain while it is tethered to the Sec61 complex (Fig. 6). US2, but not transferrin receptor and calnexin (not shown), is

also recovered in a complex with Sec61 (Fig. 5a). Interestingly, it is predominantly the non-glycosylated form of US2 that is coprecipitated, consistent with the suggestion that US2 escorts class I heavy chains on their way to destruction.

### A complex of Sec61 and class I heavy chains

Class I heavy chains are also degraded in normal cells if they fail to assemble properly<sup>20,26</sup>, a process that probably occurs in the cytosol because it can be partially inhibited by proteasome inhibitors (data not shown). Degradation of class I HC is greatly stimulated if their misfolding is induced by DTT (Fig. 2). To test the possible involvement of the Sec61 complex in the degradative pathway, co-immunoprecipitation experiments were done (Fig. 5). In the absence of DTT, association of class I molecules with Sec61 was only seen at late times of the chase period. In the presence of DTT, the association occurred earlier and was more pronounced. These data therefore suggest that even in normal cells, unfolded class I heavy chains interact with the Sec61 complex before being degraded in the cytosol. We detected only the glycosylated heavy chain in a complex with Sec61. Although the formation of a complex of class I heavy chains with Sec61 precedes their proteolytic destruction and suggests that Sec61 is involved, the absence of a deglycosylated class I HC in association with Sec61 indicates that the rate-limiting steps for degradation of class I HC in control and in US2<sup>+</sup> cells must differ.

### Discussion

HCMV uses a remarkable mechanism to downregulate the expression of the MHC class I molecules in infected cells. Two viral gene products, US11<sup>5</sup> and—as shown here—US2, induce newly synthesized MHC class I molecules to be transported back

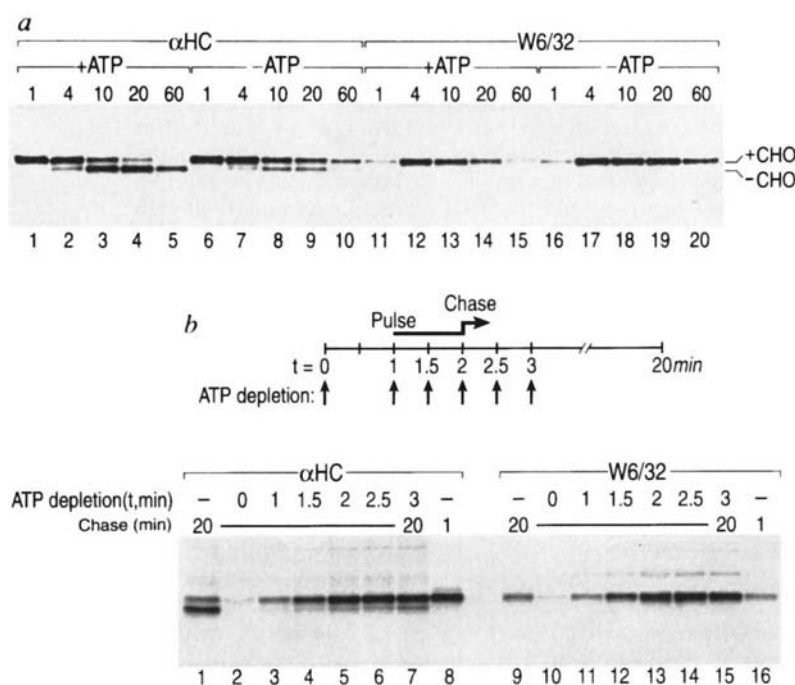


FIG. 3 The dislocation of MHC class I heavy chains is ATP-dependent. **a**, US2<sup>+</sup> cells treated with ZL<sub>3</sub>H were pulse-chased in the presence (–ATP) and absence (+ATP) of 2-deoxyglucose and antimycin A. Folded class I molecules were recovered with W6/32, and free heavy chains with anti-HC. Immunoprecipitates were resolved by SDS–PAGE. In the absence of ATP, the decreased conversion of the glycosylated precursor (+CHO) to the deglycosylated product (–CHO) is compensated by an increase in properly folded (W6/32-reactive) class I molecules. **b**, Pulse–chase experiment on US2<sup>+</sup> cells in the presence of ZL<sub>3</sub>H, in which all samples were labelled and chased for the same times, with the ATP-depletion mixture added at the indicated times (see scheme). Addition of ATP-depletion mix at the onset of labelling completely suppresses the conversion of glycosylated heavy chain to the deglycosylated intermediate.

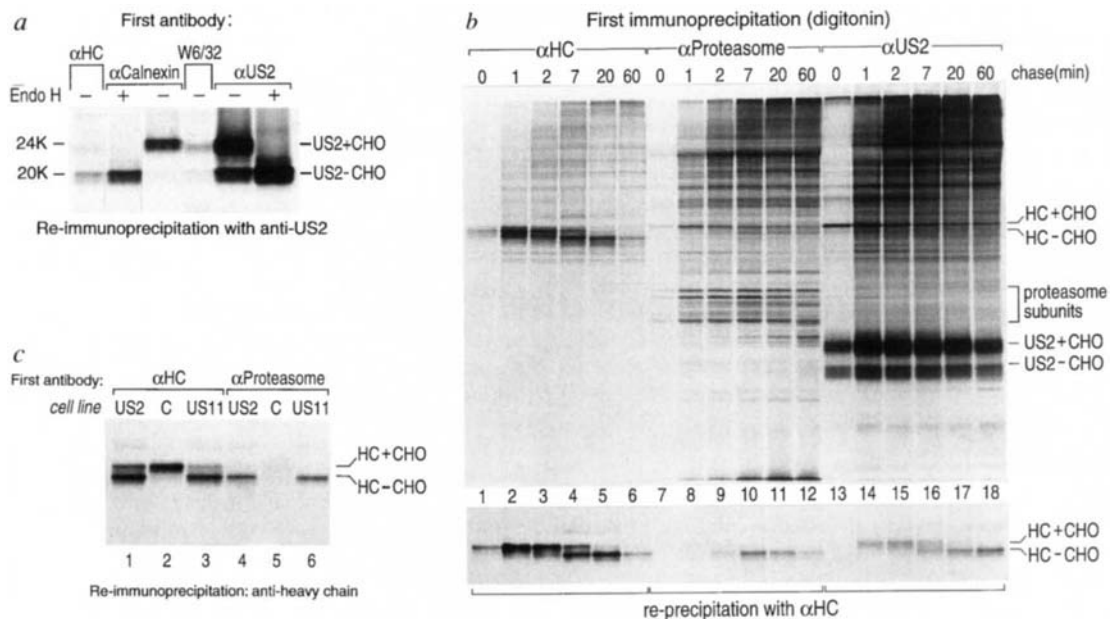


FIG. 4 US2 associates with MHC class I heavy chains and the breakdown intermediate occurs as a soluble intermediate complexed with the proteasome. *a*, Glycosylated (US2 + CHO) US2 associates with calnexin and properly folded class I molecules (W6/32), whereas non-glycosylated US2 (US2 - CHO) is found in a complex with the class I heavy chain breakdown intermediate. Glycosylation status was assessed by digestion with EndoH, as indicated. *b*, Digitonin lysates of ZL<sub>3</sub>H treated US2<sup>+</sup> cells were immunoprecipitated by the antibodies indicated and the precipitates

were reimmunoprecipitated with rabbit anti-heavy chain serum (bottom panel). The positions of migration of the heavy chains (HC) and US2 molecules (US2) with or without their *N*-linked glycan ( $\pm$ CHO) are indicated. *c*, Deglycosylated intermediate (HC - CHO) is found in association with the proteasome in digitonin extracts of ZL<sub>3</sub>H treated US2<sup>+</sup> and US11<sup>+</sup> cells, but not control cells, even though class I heavy chains are present in all cell lines.

from the ER into the cytosol. Following deglycosylation they are degraded by the proteasome. Using US2-expressing cells, we have been able to elucidate the mechanism of this process.

US2 interacts directly with newly synthesized class I molecules and escorts them back into the cytosol in a reaction that involves the Sec61 complex and therefore appears to be the reversal of the translocation process that initially deposited them into the ER. Misfolded or unassembled class I heavy chains in normal cells seem to be routed in a similar pathway for their destruction in the cytosol.

The Sec61 complex was thought to have a role only in the translocation of proteins from the cytosol into the ER. It is an essential translocation component that can associate with either ribosomes or the Sec62/63 complex to perform co- and post-translational transport, respectively<sup>7,27-29</sup>. The Sec61 complex forms a protein-conducting channel while the driving force for transport is provided by other, interacting components. There is no reason to assume that this channel must allow transport in only one direction, and one could therefore envisage that the channel may indeed be used for retrograde transport if associated with appropriate additional components. Retrograde transport *in vitro*, presumably through the Sec61 channel, has been reported<sup>30-32</sup> but its physiological significance is unclear. Our conjecture that retrograde transport through the Sec61 channel is used for cytosolic degradation of ER proteins is based on the specific association of class I HC breakdown intermediates with the Sec61 complex at times when degradation proceeds at maximum rate. To our knowledge, our data provide the first indication that polypeptide chains can be caught in transit through the channel.

Reversal of the translocation process would be easier to explain if the newly synthesized polypeptide chain had never left the Sec61 channel. Indeed, this may be true for many class I HC in US2<sup>-</sup> or US11-expressing cells, because their degradation occurs so soon after membrane insertion (Fig. 6*a*). However, in US2-expressing cells, dislocation into the cytosol is possible even for folded class I molecules, which therefore have probably left the translocation site. We postulate that membrane proteins can be brought back

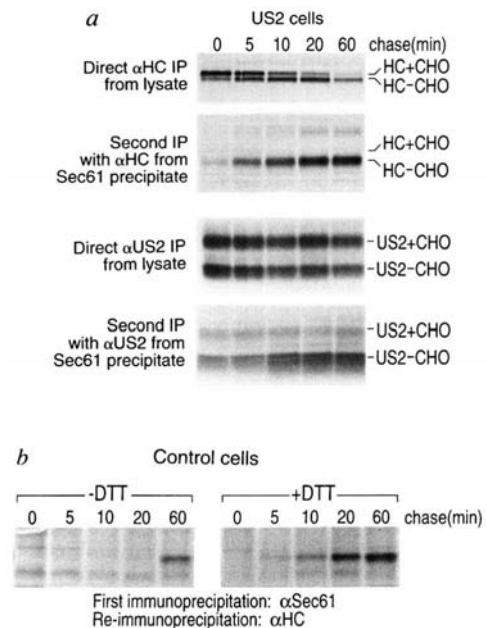
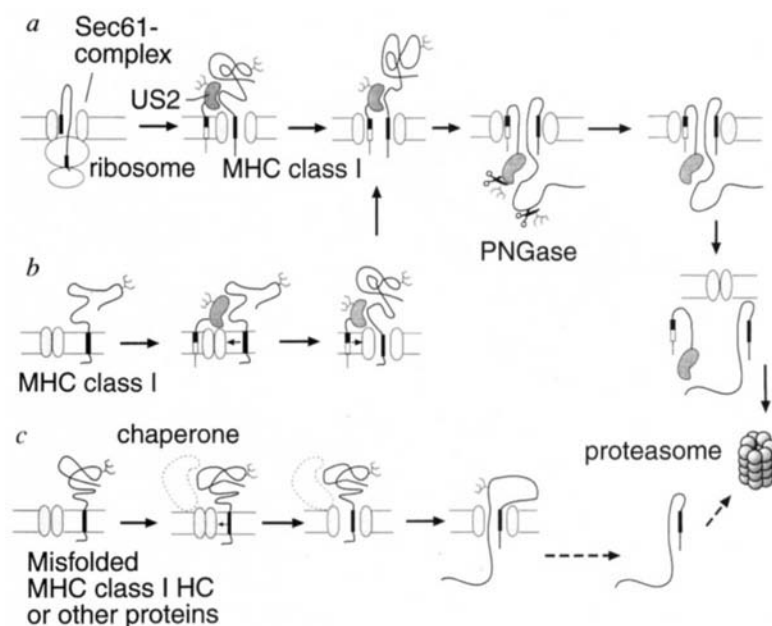


FIG. 5 Class I molecules destined for degradation associate with Sec61p. *a*, Sec61 was immunoprecipitated from digitonin lysates of ZL<sub>3</sub>H-treated US2<sup>+</sup> cells and the precipitates were reimmunoprecipitated with either rabbit anti-HC serum ( $\alpha$ HC) and/or rabbit anti-US2 serum ( $\alpha$ US2). Sec61-depleted lysates were also immunoprecipitated with  $\alpha$ HC or  $\alpha$ US2. These samples are designated as direct immunoprecipitates. The positions of migration of the heavy chains (HC) and US2 molecules (US2) with or without their *N*-linked glycan ( $\pm$ CHO) are indicated. Exposure time of the autoradiogram for the reprecipitation experiments was 14 days; for direct precipitations, it was 2 days. *b*, Control cells were chased in the absence or presence of DTT, as indicated. Sec61 was immunoprecipitated as for *a*. These immunoprecipitates were reimmunoprecipitated with  $\alpha$ HC. Protein misfolding promoted by DTT results in an increased representation of glycosylated class I HC in the Sec61 complex.

FIG. 6 Model for Sec61-dependent dislocation. **a**, A nascent class I heavy chain inserts into the ER in a signal sequence-dependent fashion. US2 interacts with the class I heavy chains before their lateral escape from the Sec61 complex into the ER membrane. The luminal, glycosylated domains of both class I HC and US2 are subject to dislocation, a reaction that culminates in the action of *N*-glycanase and proteasomal proteolysis. **b**, An ER-resident, properly folded class I HC can be recruited by US2 to re-enter the Sec61 complex. US2 itself re-enters the Sec61 complex and both polypeptides are dislocated. **c**, In normal cells, any misfolded or incompletely assembled protein could be recruited by the action of an ER-resident chaperone to re-engage the Sec61 complex for dislocation and cytosolic destruction. Whereas in **a** and **b**, proteolysis may become rate-limiting owing to the high rate of dislocation, in **c** dislocation is the rate-limiting step.



into the translocation channel, perhaps by reversal of the lateral gating mechanism that led to their release into the phospholipid bilayer in the first place. Because glycosylated US2 associates with glycosylated class I molecules, we believe that it is this association that triggers the re-entry of the heavy-chain molecules into the translocation channel (see model in Fig. 6b).

The next step in the US2-dependent breakdown of the heavy chains is deglycosylation by a cytosolic *N*-glycanase<sup>5,25</sup>. The resulting class I HC intermediate is associated with non-glycosylated US2, which suggests that they are dislocated in a complex. The association of both deglycosylated class I HC and US2 with the Sec61 complex strengthens this proposal. We envisage that the luminal domains of class I HC and US2 are threaded into the channel so that they achieve loop structures similar to those formed during their insertion into the ER (Fig. 6). The occurrence of the deglycosylated class I HC in association with Sec61 implies that the luminal domain of the class I HC must be accessible to the cytosolic *N*-glycanase while still tethered to Sec61.

The final step in the degradation pathway is the proteasome. Inhibition of degradation by proteasome inhibitors is required to visualize the class I HC intermediate, and for the detection of a complex between the proteasome and the intermediate in US2-expressing cells. A complex between the proteasome and a substrate is without precedent and may be detectable in US2<sup>+</sup> cells only because of the unusually high rate of dislocation of MHC molecules. We have been unable to detect ubiquitin-conjugated class I HC in the course of pulse-chase experiments, perhaps because such intermediates are too short-lived, but the ability of the 26S proteasome to attack non-ubiquitylated proteins is on record<sup>33–35</sup>.

Most of the substrates of the proteasome are soluble, deglycosylated class I HC, but those still located in the translocation channel may also be attacked by a population of proteasomes that is membrane-bound<sup>36</sup>. Indeed, in mouse epithelial cells engaged in the breakdown of misfolded human class I molecules, the ubiquitin-activating enzyme E1 and ubiquitin itself were found associated with an expanded compartment to which the misfolded class I heavy chains were targeted<sup>37</sup>. Although ubiquitylation may be involved only in US2-independent degradation, membrane association of the proteasome may be more generally involved in disposal of dislocated proteins.

Whether the degradation pathway of MHC class I molecules in normal cells is identical to that in US2<sup>+</sup> cells is not entirely clear, because proteasome inhibitors did not block degradation completely and no deglycosylated breakdown intermediate could be

detected as yet. The latter may simply be too short-lived. If the rate-limiting step in the overall pathway is the feeding of a polypeptide into the translocation site for dislocation, it may be difficult to detect breakdown intermediates. For those proteins where ER breakdown has been invoked, their half-lives are on the 15–180-min timescale<sup>9</sup> without the occurrence of obvious breakdown intermediates. Only if the dislocation step is greatly accelerated, as in the case of US2- or US11-expressing cells, and if proteasome function is inhibited, may it be possible to catch intermediates.

Regardless of whether the later steps are the same in US2<sup>+</sup> and control cells, our results suggest that dislocation occurs by the same mechanism. MHC class I molecules that are induced to misfold in the ER are associated with the Sec61 complex at the time point of their maximum degradation. Misfolded heavy chains may associate with ER-resident chaperones<sup>38</sup> that, analogously to US2 and US11, bring the polypeptides back into the translocation channel for dislocation into the cytosol and subsequent degradation (Fig. 6). The postulated chaperones need not be homologous with US2 or US11 as these are also not related to each other<sup>39</sup>, and still induce the same process. As far as we have been able to determine, the effects of US2 and US11 are specific for MHC class I molecules<sup>5</sup>, whereas the putative chaperones would have broader substrate specificity. Because the structures of US2 and US11 are quite different<sup>39</sup>, they may either target unique determinants of the translocation/dislocation machinery, as suggested by their differential attack on murine class I HC, or they may interact with different regions of class I HC (R. P. Machold and H.L.P., unpublished observations), while otherwise exploiting a similar mechanism of dislocation.

We propose that the pathway outlined in Fig. 6c is generally used to purge the ER of misfolded or unassembled proteins. If some proteins can be dislocated back into the cytosol, there must be a mechanism that distinguishes them from those that stay in the ER or are transported further along the secretory pathway. With few exceptions, type I and type II membrane proteins occur in homo-oligomeric or hetero-oligomeric forms early in their biosynthesis. The original concept of architectural editing encompassed a check on proper oligomeric state as one of the criteria for quality control<sup>40</sup>. Failure to oligomerize would leave exposed those areas of the protein involved in intersubunit contacts, which could now be used to allow an interaction, either directly, or via the above-mentioned chaperones, with the Sec61 complex (Fig. 6c). Such a model bears obvious similarities with cytosolic proteolysis, where recognition of unfolded proteins by chaperones



precedes degradation by cytosolic proteases such as the proteasome<sup>15,41</sup>.

ER degradation is a long known phenomenon but was hitherto assumed to occur in an ATP-dependent manner<sup>42</sup> within the membrane or in a luminal compartment of the ER or of an ER derivative. ATP may be required both for the dislocation step that precedes cytosolic destruction and for proteasomal proteolysis<sup>15,43</sup>. It becomes increasingly clear that many soluble and membrane-bound proteins in yeast<sup>44</sup> and in mammals<sup>45–47</sup> are degraded in the cytosol. Our results suggest that the Sec61 channel may provide the general pathway by which retrograde transport to the cytosol occurs. □

## Methods

**Antibodies and inhibitors.** The following antibodies were used: anti-proteasome (Organon Teknica, Oss, The Netherlands); anti-Sec61 $\beta$ <sup>24</sup>; monoclonal antibody W6/32<sup>16</sup>; rabbit anti-heavy chain serum (RaHC)<sup>5</sup>; and rabbit anti-US2 serum (T.R.J. and L. Sun, manuscript submitted). The proteasome inhibitor lactacystin<sup>17</sup> was obtained from E. J. Corey. Carboxybenzyl-leucyl-leucyl-leucinal<sup>48</sup> (ZL<sub>3</sub>H) was synthesized as described<sup>5</sup>. The synthesis of carboxybenzyl-leucyl-leucyl-leucyl vinylsulfone (ZL<sub>3</sub>VS), a compound that covalently and specifically modifies the proteasomal  $\beta$ -subunits will be reported elsewhere. Antimycin A, 2-deoxyglucose, and tunicamycin were from Sigma; digitonin was from Calbiochem.

**Pulse-chase experiments.** U373-MG astrocytoma cells used as control cells, US2 transfectants (US2<sup>+</sup>) and US11 transfectants (US11<sup>+</sup>)<sup>4,49</sup> were maintained and used for pulse-chase experiments essentially as described<sup>5</sup>. Procedures for preparation of cell lysates using NP-40 lysis mix and immunoprecipitation have been described<sup>5,20</sup>.

**Gel electrophoresis.** SDS-polyacrylamide gel electrophoresis (SDS-PAGE), one-dimensional isoelectric focusing (IEF) and fluorography were performed as described<sup>50</sup>.

**Subcellular fractionation of a pulse-chase experiment of US2<sup>+</sup> cells.** A pulse-chase experiment was performed at 37 °C on US2<sup>+</sup> cells labelled in the presence of ZL<sub>3</sub>H (20  $\mu$ M). The cells were pulsed with 400  $\mu$ Ci [<sup>35</sup>S]methionine for 1 min and chased for 0, 1, 5, 10, 20 and 40 min. The subcellular fractionation and subsequent immunoprecipitation were done as described<sup>5</sup>.

**Pulse-chase experiment including DTT during the chase.** A pulse-chase experiment was done at 37 °C with control and US2<sup>+</sup> cells in the presence of

ZL<sub>3</sub>H (20  $\mu$ M). The cells were pulsed with 200  $\mu$ Ci of [<sup>35</sup>S]methionine for 10 min and chased for 0, 20, 40 and 120 min. Dithiothreitol (DTT) was added at a final concentration of 2 mM at 7 min into the pulse. Where indicated, DTT was washed out by the addition of 50 ml of culture medium. The cells were lysed in NP-40 lysis mix and class I molecules were immunoprecipitated with RaHC or W6/32. The proteins were separated by SDS-PAGE.

**ATP depletion of US2<sup>+</sup> cells.** A pulse-chase experiment was done at 37 °C with US2<sup>+</sup> cells in the presence of ZL<sub>3</sub>H (20  $\mu$ M). The cells were pulsed with 50  $\mu$ Ci [<sup>35</sup>S]methionine for 1 min. Excess non-radioactive methionine was added simultaneously with ATP-depletion mix<sup>22</sup>, comprising 2-deoxyglucose and antimycin A to final concentrations of 22.5 mM and 13.5  $\mu$ M, respectively. Cells were chased for 0, 1, 4, 10, 20 and 60 min. Alternatively, ATP-depletion mix was added before, during or after a 1-min pulse, followed by a chase, so that all samples were chased for 20 min (Fig. 3b). Class I molecules were recovered with either RaHC or W6/32. Proteins were separated by SDS-PAGE.

**Re-immunoprecipitation of class I heavy chains from immune complexes containing proteasomes or US2.** US2<sup>+</sup> cells were pulsed for 45 s with 200  $\mu$ Ci of [<sup>35</sup>S]methionine and chased for 0, 1, 2, 7, 20 and 60 min in the presence of ZL<sub>3</sub>H (20  $\mu$ M) at 37 °C. Cells were lysed in a digitonin lysis mix (1% digitonin in 2.5 mM HEPES, pH7.6, 10 mM CaCl<sub>2</sub>). MHC class I molecules were immunoprecipitated from SDS-dissociated immune complexes prepared in a first round of immunoprecipitation with RaHC, anti-proteasome, or anti-US2 antibodies. Proteins were separated by SDS-PAGE.

**Re-immunoprecipitating class I heavy chains and US2 from Sec61 immune complexes.** A pulse-chase experiment was done at 37 °C with control and US2<sup>+</sup> cells in the presence of ZL<sub>3</sub>H (20  $\mu$ M). Cells were pulsed with 100  $\mu$ Ci of [<sup>35</sup>S]methionine for 10 min and chased for 0, 5, 10, 20 and 60 min. To one set of samples, a final concentration of 2 mM DTT was added 7 min into the pulse. Cells were lysed with a digitonin lysis mix and anti-Sec61 $\beta$  was used to immunoprecipitate the Sec61 complex from the cell lysates. The precipitated complexes were dissociated with SDS and suspended in NP-40 lysis mix. Sequential immunoprecipitations with RaHC and anti-US2 were carried out. Heavy chains and US2 molecules were also recovered from Sec61-depleted cell lysates with RaHC or anti-US2. Proteins were separated by SDS-PAGE.

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# Decrease in the space density of quasars at high redshift

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QUASARS have been found up to redshifts of almost five, corresponding to an epoch when the Universe was less than ten per cent of its present age. This leaves a rather short time for the formation of the galaxies in which quasars are believed to be embedded. Indeed, some optical studies<sup>1-3</sup> indicate that the space density of quasars does decline rapidly for redshifts  $z > 3$ , as expected if this is the epoch of galaxy formation. The interpretation of this decline is equivocal, however, as it could result simply from the obscuration of distant quasars by dust in intervening galaxies<sup>4</sup>. Radio emission from quasars, on the other hand, is unaffected by dust, and we show here that the space density of radio-loud quasars also decreases strongly for  $z > 3$ , demonstrating that the decline is real, at least for these objects. We argue that this conclusion probably applies to all quasars. If quasars are associated with galaxy formation and/or interactions between galaxies, the decline in their space density at high redshift provides a measure of the timescale for the onset of these processes.

Our search for radio-loud high-redshift quasars was based on a large sample of flat-spectrum Parkes radio sources from a survey region covering 40% of the sky (flat spectrum here means spectral index  $\alpha > -0.4$  between wavelengths  $\lambda$  of 11 and 6 cm, where flux density  $S_\nu \propto \lambda^{-\alpha}$ ). Although only a small fraction of quasars are radio-loud, it is possible to isolate quasar candidates very efficiently from radio surveys covering large areas of sky: of the flat-spectrum sources in the Parkes catalogue which were already optically identified, 83% are quasars. Quasars at very high redshifts ( $z > 5$ ) can be distinguished by the fact that they should be heavily obscured in the optical B band by intervening hydrogen, because the Lyman limit is redshifted past this band, and they will only be detectable at very red wavelengths. Thus, our strategy was to look for extremely red stellar objects at the positions of flat-spectrum radio sources.

There are two critical assumptions. (1) Very high-redshift quasars will still have flat spectra between the observed wavelengths 11 and 6 cm. But spectra which are flat at the usual centimetre wavelengths are often steeper at shorter wavelengths; at sufficiently high redshifts this steep portion of the spectrum will be redshifted into the window between 11 and 6 cm where the sample is selected, so that very high-redshift quasars could be excluded in the selection process. We have determined the redshift at which this becomes important by examining the quasi-simultaneous radio spectra of a sample of quasars and BL Lac objects measured by Gear *et al.*<sup>5</sup>. For each object we measured the wavelength at which the spectrum becomes steeper than  $\alpha = -0.4$ , and determined the redshift at which this point in the spectrum would appear at an observed wavelength of 6 cm. We find that our first assumption is valid at least up to  $z \approx 10$ ; median values are  $z \approx 27$  and  $39$  for quasars and BL Lacs respectively. (2) The second assumption, that  $z > 5$  quasars will be obscured in the optical B band, is justified on the basis of both the properties of observed quasars and simulations based on extrapolations of the known statistics of intervening hydrogen absorbers to higher

redshifts. Good-quality spectra<sup>6</sup> of quasars at  $4 < z < 5$  illustrate the abrupt cutoff in flux at wavelengths less than the Lyman limit. At  $z = 5$  the Lyman limit occurs at a wavelength of 5,472 Å, to the red of the B band, which should therefore be heavily obscured. The statistical properties of the Lyman- $\alpha$  forest lines and Lyman-limit systems, and their dependence on redshift, have been studied by several authors<sup>6,7</sup>. On the basis of these properties, simulations can be made<sup>8-10</sup> which permit extrapolations to higher redshifts. Figure 1 shows the expected absorption in the B band; 10 mag is typical even at  $z = 5$ .

Our procedure, then, was to select all sources from the updated Parkes catalogue<sup>11</sup> with spectral indices between 11 and 6 cm flatter than  $-0.4$ , in the declination range  $+2.5^\circ$  to  $-80^\circ$ . We excluded low galactic latitudes ( $|b| < 10^\circ$ ), and regions around the Magellanic Clouds (right ascension 00–02 h, declination  $-69^\circ$  to  $-76^\circ$ ; right ascension 04 h 30 min to 06 h 30 min, declination  $-64^\circ$  to  $-75^\circ$ ). Of these 878 sources, only 229 were listed in the catalogue as unidentified, and these were our potential candidates for  $z > 5$  quasars. (We return to the 'identified' sources below). We obtained radio positions accurate to better than 1 arcsec for these unidentified sources, half from new VLA or Australia Telescope measurements, and the others from previously published and unpublished measurements (the complete data set and details of our radio and optical observations will be presented elsewhere). These accurate positions were then cross-correlated against the COSMOS catalogue of the southern sky, which is based on the UK Schmidt J survey with a limiting magnitude of  $B_J \sim 22.0$ – $22.5$ . After correction for systematic reference-frame errors, the scatter in the radio–optical positional differences was 0.88 arcsec r.m.s. for both quasars and galaxies, and 3 arcsec was taken as the cutoff for identifications. This cross-correlation produced another 128 identifications.

To identify the remaining sources, sensitive observations using charge-coupled devices (CCDs) were necessary. We used the ESO Faint Object Spectrograph and Camera (EFOSC) at the Cassegrain focus of the 3.6-m telescope at La Silla, and observed in the B, Gunn-i and Gunn-z filters (wavelength ranges 3,700–5,200 Å, 7,100–9,100 Å, and 8,200–10,500 Å, respectively). Any  $z > 5$  quasar would be expected to show up as a very red stellar object at the radio position, present in Gunn-z but not in B.

All of the unidentified sources have now been identified, either as galaxies, or as stellar objects which are present in the B band

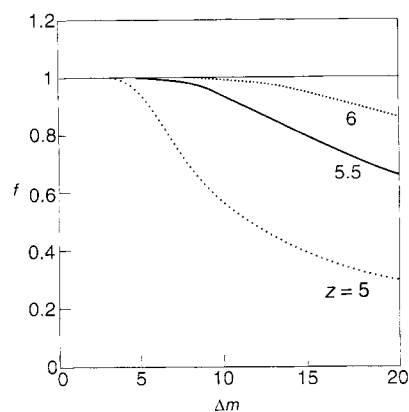


FIG. 1 Expected fraction  $f$  of quasars at redshifts 5, 5.5 and 6 for which the absorption in the B band due to the Ly $\alpha$  forest and Lyman limit is greater than  $\Delta m$  magnitudes, as a function of  $\Delta m$  (based on calculations by P. Møller (personal communication; Møller and Warren<sup>9</sup>)). These extrapolations assume  $dn/dz \propto (1+z)^{2.75}$  and  $f(N)dN \propto N^{-1.60}$  where  $n$  is the number-density of Lyman absorbers and  $N$  is the column-density of neutral hydrogen due to these absorbers.

(and therefore not at  $z > 5$ ). For the 24 stellar objects identified from CCD observations the B magnitude criterion was supplemented by spectroscopy and/or colour measurements. The one outstanding source was PKS1251–407, identified with a very red stellar object, although because it was still faintly visible in B it was not considered a  $z > 5$  candidate. Indeed, it was found to have a redshift of 4.46, making it the highest-redshift radio-loud quasar known<sup>12</sup>. Objects at higher redshifts could have been found with similar ease. Thus, as all sources are identified, there are none left which could be quasars at  $z > 5$ .

Could any  $z > 5$  quasars still be hiding amongst the sources identified on the Schmidt plates—those listed as identified in the catalogue, or those identified from the COSMOS database? We tested the reliability of the catalogued identifications by checking half of them using new accurate radio positions; we re-identified 97% of them in  $B_r$ , showing that the original identifications are highly reliable. Their very presence on  $B_r$  Schmidt plates makes them too bright to be at  $z > 5$ . Even the most luminous quasars known would be fainter than the UK Schmidt plate limit if they were located at  $z > 5$  behind just 5–6 magnitudes of absorption (which is at the low end of the distribution in Fig. 1). Furthermore, about half of the identified quasars in the Parkes catalogue already have measured redshifts, and all are below 4. And finally, none of the  $B - R$  colours obtained for a random sub-sample of almost 200 of these objects is as red as expected for a  $z > 5$  quasar. For all of these reasons, it appears highly unlikely that any of the sources identified on the Schmidt plates are at  $z > 5$ . As the same applies to the sources identified from the CCD observations, we conclude

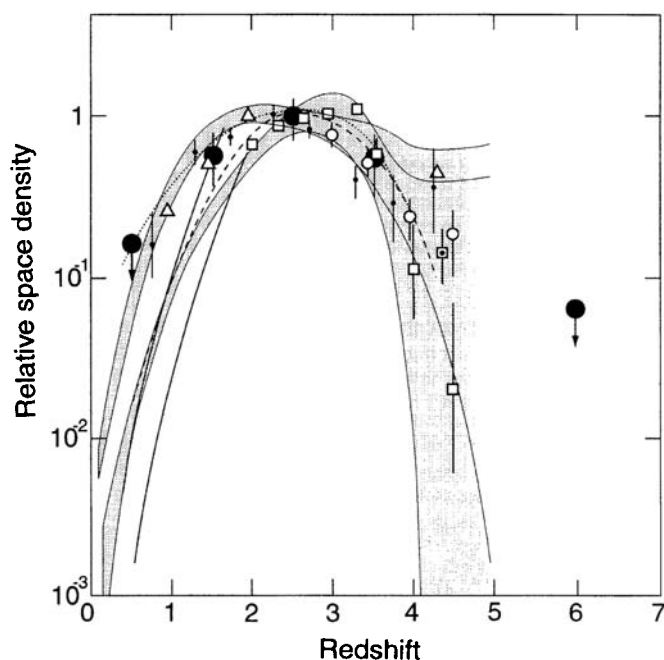


FIG. 2 Space densities, co-moving, normalized to  $z \approx 2-3$  and plotted as a function of redshift, for the Parkes flat-spectrum radio-loud quasars with  $P_{11} \geq 1.1 \times 10^{27} \text{ W Hz}^{-1} \text{ sr}^{-1}$  (large filled circles). The number of such objects found in the individual redshift ranges are 0 ( $0 < z < 1$ ), 7 ( $1 < z < 2$ ), 12 ( $2 < z < 3$ ), 6 ( $3 < z < 4$ ) and 0 ( $5 < z < 7$ ); the error bars correspond to  $\pm\sqrt{N}$  and the upper limits to one object. For comparison, similarly normalized space densities are also shown for the optically-selected quasar samples of Warren *et al.*<sup>1</sup> (empty squares), Schmidt *et al.*<sup>2</sup> (empty circles), Kennefick *et al.*<sup>3</sup> (dotted square), Hawkins & Véron<sup>26</sup> (small filled circles) and Irwin *et al.*<sup>27</sup> (combined and normalized with Miller *et al.*<sup>28</sup> (empty triangles)), the flat- and steep-spectrum sources studied by Dunlop and Peacock<sup>29</sup> (shaded areas), and the flat-spectrum radio-loud quasar sample of Hook *et al.*<sup>30</sup> (dotted line). The solid lines represent lower-redshift luminosity functions<sup>31,32</sup> as used by some of the above authors. The dashed line is a representative curve used in Fig. 3.

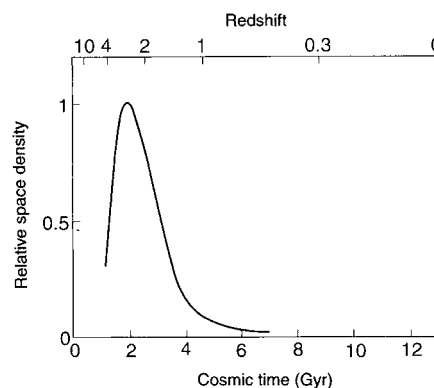


FIG. 3 Schematic representation of the relative space density of quasars as a function of cosmic time, computed from the dashed curve in Fig. 2.

that none of the 878 sources in our original sample is a quasar at  $z > 5$ .

This enables us to place an upper limit on the space density of flat-spectrum radio-loud quasars at  $z > 5$  which is independent of optical magnitude. The limiting flux density of the Parkes catalogue varies over the sky, so for simplicity in computing this space density we consider only those areas which have been completely surveyed down to a single well defined limit, which we take to be 0.25 Jy at 11 cm. The absence of any such quasars in the co-moving volume covered by this 3.8-sr area and the redshift range  $5 < z < 7$  can be directly compared with measured space densities in lower-redshift bins of quasars with radio luminosities  $P_{\text{lim}} \geq 1.1 \times 10^{27} \text{ W Hz}^{-1} \text{ sr}^{-1}$  corresponding to flux densities  $\geq 0.25 \text{ Jy}$  at  $z = 7$  ( $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$  and  $q_0 = 0.5$  are used throughout this Letter). In determining these lower redshift space densities we used a restricted declination range of the Parkes catalogue ( $+2.5^\circ$  to  $-45^\circ$ ), in which the fraction of quasars with measured redshifts is high (96% for 11-cm flux density  $S_{11} > 0.5 \text{ Jy}$ ). As we can use only sources with known redshifts, the space densities we compute at  $z < 5$  are lower limits, to be compared with the upper limit at  $z > 5$ .

These co-moving space densities, normalized to  $z \approx 3$ , are plotted against redshift in Fig. 2. (Although  $q_0 = 0.5$  is used here, a similar result is also found for  $q_0 = 0.1$ .) The decrease at high redshift is obvious; considering that 25 quasars above  $P_{\text{lim}}$  are found at  $z < 4$  and none at  $5 < z < 7$ , the turnover is significant at the 99.96% level (99.98% when we use our entire sample, including zones of the Parkes catalogue complete to 0.20 and 0.65 Jy). If there were no fall-off in the co-moving space density of quasars at high redshift, we would expect to find at least 15 quasars at  $5 < z < 7$  in our sample.

This absence of high-redshift quasars in our sample is very unlikely to be due to obscuration for the following reasons. (1) Radio emission is unaffected by dust, and all sources are already accounted for—there are simply none left to be  $z > 5$  quasars. This excludes the possibility of obscuration by intervening galaxies or dusty Lyman-limit systems too faint to be seen directly. (2) The possibility that some of the sources are in fact at  $z > 5$  but have been misidentified with visible foreground galaxies that obscure them cannot explain the turnover, because it would require accurate positional coincidences of all of the  $z > 5$  quasars with observed foreground galaxies. The probability of that is negligible, about  $10^{-15}$ . Furthermore, gravitational bending of the light from the quasar by the intervening galaxy would generally increase the impact parameter and decrease any obscuration<sup>13</sup>. (3) Another possible source of obscuration at high redshifts is Thomson scattering by an ionized intergalactic medium, which affects all frequencies  $\ll mc^2/h$  equally. However, even if all the baryons in the Universe were distributed in an ionized intergalactic plasma with  $\Omega_b$  at the improbably high value of 0.5, the effect on the



results shown in Fig. 2 would still be small. (4) The possibility that some sources have been completely missed (also in the radio) due to both optical obscuration and radio free-free absorption in intervening objects is highly implausible, because a large free-free optical depth would imply a small size for any reasonable excitation parameter, hence a very small probability of a line-of-sight coincidence. (5) Obscuration by large amounts of dust and ionized gas intrinsic to the quasar is conceivable, but this does not affect the conclusions drawn here. We are concerned with a particular observed phenomenon, that is, quasars similar to those seen at  $z \sim 1-2$ , and the space density of such objects decreases at high redshifts. (There must obviously be precursors at still higher redshifts, at least in the form of mass concentrations, but that is a different issue.) Furthermore, even if there are 'hidden' quasars enshrouded in dust and ionized gas, if quasars are short-lived objects we would expect such hidden quasars to exist at all redshifts, so the redshift turnover would be unaffected. For all of these reasons, it is concluded that the observed turnover in the space density of flat-spectrum radio-loud quasars is real, and not due to obscuration.

Can it now be argued that this result applies to all quasars, and not just to the flat-spectrum radio-loud quasars? The idea of a 'redshift cutoff' or turnover in the space density of quasars goes back several decades, and the strongest evidence has come from recent optically selected samples<sup>1-3</sup>, but it has been proposed that any turnover in optically selected samples could be due to obscuration by dust<sup>4</sup>. Several arguments suggest that this is not the case. (1) Figure 2 shows normalized space densities from several other studies, and the overall agreement in the turnover is striking, even though the different samples pertain to different luminosities and classes of objects. It is unlikely to be mere coincidence that the radio and optical quasar populations turn over together. (2) The fraction of optically selected quasars which are flat-spectrum radio sources appears to be roughly constant with redshift at  $z > 1$  (ref. 14), and there is no theoretical reason as to why it should change with redshift. (3) If the turnover in optically selected samples were due to obscuration, radio quasars would be similarly affected. Thus, if the apparent space density were down by a factor of, say, ten at  $z \approx 4.5$  due to obscuration, there would be only a 10% chance of our having found PKS1251-407 rather than a blank field. (4) The ultraviolet background radiation field may have a similar dependence on redshift, as would be expected if it is produced largely by the quasars; it increases by two orders of magnitude from  $z \approx 0.5$  to  $z \approx 3$  (refs 15-18), and there are some indications that it may decrease again at still higher redshifts<sup>19,20</sup>. (5) Recent studies<sup>21,22</sup> which consider the possible effects of dust in intervening galaxies and H I clouds suggest that the obscuration due to these objects would not suffice to produce the redshift turnover. It has even been suggested that many such objects at high redshift may be essentially dust-free<sup>23,24</sup>. In this context, if it is assumed that the radio fraction of quasars remains constant at the highest redshifts, then we can use the relative turnovers in the radio and optical samples in Fig. 2 to set an upper limit on the amount of obscuration due to dust. We find that the total obscuration is probably less than that in model B of Fall and Pei<sup>21</sup>, for which optical depth  $\bar{\tau}_{2-4} = 1.7$ , and well below that estimated in other similar studies<sup>4,25</sup>.

We conclude that the turnover probably does apply to all quasars, and is not strongly affected by dust obscuration. In that case, and if quasars are associated with galaxy formation and/or interaction as is widely believed, then the onset and rapid increase in their space density with cosmic time at this early epoch (Fig. 3) provides a measure of the timescale for these processes.  $\square$

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## Structural characterization of pentacoordinate silicon in a calcium silicate

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**IN silicate minerals formed at pressures typical of the Earth's crust, the silicon is usually coordinated by four oxygen atoms. In contrast, silicates formed at higher pressures, typical of the Earth's transition zone and lower mantle, contain predominantly six-coordinated silicon. Silicon coordinated by five oxygen atoms is not normally found as a structural element in crystalline phases, but is nevertheless believed to play a central role in many dynamic processes that occur in silicates. For example, pentacoordinate silicon is probably a component of aluminosilicate melts and glasses at mantle temperatures and pressures<sup>1,2</sup>, where it will dominate their transport properties<sup>3-6</sup>; it is also believed to act as an intermediate activated state during oxygen diffusion in silicate minerals<sup>7,8</sup>. Here we report the complete structure determination of an inorganic crystalline silicate—CaSi<sub>2</sub>O<sub>5</sub>—containing SiO<sub>5</sub> groups. Our results confirm the previous attribution<sup>1,2,9</sup> of peaks in the <sup>29</sup>Si NMR spectrum of this material to the presence of pentacoordinate silicon, and the detailed geometry that we determine for the SiO<sub>5</sub> group should**

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provide a firm basis for characterizing and quantifying the role of pentacoordinate silicon in silicate melts and glasses.

Phases with  $\text{CaSi}_2\text{O}_5$  stoichiometry synthesized at high pressures and temperatures were first reported by Kanzaki and co-workers<sup>9</sup> who, on the basis of the observation of an NMR peak at a chemical shift of  $-150$  p.p.m., suggested that one phase contained pentacoordinate silicon. We synthesized a high-pressure phase of  $\text{CaSi}_2\text{O}_5$  from a glass of the same composition loaded into a rhenium capsule and held at 11 GPa and  $1,350^\circ\text{C}$  for 4 hours in the 1,000 ton multi-anvil press at the Bayerisches Geoinstitut, Bayreuth. These conditions fall within the stability field of the assemblage  $\text{Ca}_2\text{SiO}_4 + \text{CaSi}_2\text{O}_5$  reported for the  $\text{CaSiO}_3$  system<sup>9-11</sup>, and the stability field of the 'titanite-like phase' of  $\text{CaSi}_2\text{O}_5$  described by Kanzaki<sup>12</sup>. The X-ray powder diffraction pattern of the recovered material exhibited some similarities to that of titanite ( $\text{CaTiSiO}_5$ ) as noted previously<sup>9</sup>, but with a large number of extra reflections. Most of these peaks could be indexed on a cell derived from that of titanite by a doubling of the  $a$  cell parameter and a relaxation of the monoclinic symmetry of the aristotype structure (Table 1). Other peaks were attributed to a trace amount of stishovite (high-pressure phase of  $\text{SiO}_2$ ), and two weak peaks were attributed to an as-yet unidentified phase.

X-ray intensity data were obtained from several crystals selected from the run products. All diffraction maxima could be indexed on the unit cell obtained from the powder diffraction data, although many crystals were twinned. The twin law corresponds to the two-fold symmetry operator that would be present were the phase to be monoclinic rather triclinic. From one untwinned crystal we were able to obtain intensity data and solve the structure (Table 1) which is very similar to that recently described in a preliminary report<sup>13</sup> except for a difference in space group. An extensive examination of the sample by transmission electron microscopy revealed that it contains a few curvilinear faults oriented approximately parallel to (100), but of such low density that their presence will not have affected the X-ray diffraction intensities.

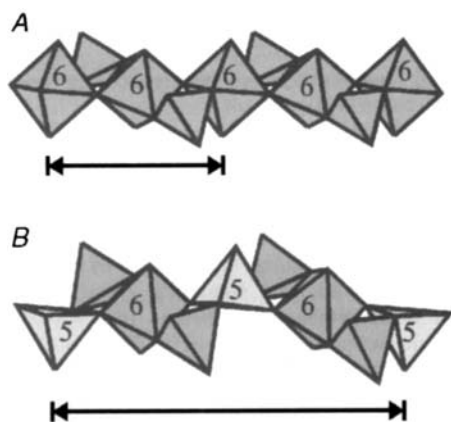


FIG. 1 Polyhedral representation of the polyhedral chains which run parallel to the crystallographic  $a$ -axis in high-temperature titanite<sup>14</sup> (A) and  $\text{CaSi}_2\text{O}_5$  (B). The coordination of the Ti atoms in A and the Si atoms in B are indicated by the arabic numerals. The alternation of 5- and 6-coordinated Si in the structure of  $\text{CaSi}_2\text{O}_5$  results in a doubling of the length of the  $a$ -cell parameter relative to that of titanite, as indicated. All of the tetrahedra shown are occupied by silicon.

TABLE 1 Crystallographic data for  $\text{CaSi}_2\text{O}_5$

| Site | Occ. | x             | y             | z             | $B_{\text{eq}}$ |
|------|------|---------------|---------------|---------------|-----------------|
| Ao   | Ca   | 0.11140(7)    | 0.43088(9)    | 0.29330(13)   | 1.011           |
| Ax   | Ca   | 0.62141(7)    | 0.40746(10)   | 0.23243(13)   | 1.117           |
| Moo  | Si   | $\frac{1}{4}$ | $\frac{1}{4}$ | $\frac{3}{4}$ | 0.871           |
| Mox  | Si   | $\frac{3}{4}$ | $\frac{1}{4}$ | $\frac{3}{4}$ | 0.859           |
| Ma   | Si   | 0.01253(9)    | 0.23431(13)   | 0.81327(18)   | 0.885           |
| Too  | Si   | 0.36576(9)    | 0.43601(13)   | 0.24022(18)   | 0.863           |
| Tox  | Si   | 0.86854(9)    | 0.44784(13)   | 0.20955(18)   | 0.868           |
| O1o  | Si   | 0.38070(22)   | 0.31432(32)   | 0.76147(46)   | 0.982           |
| O1x  | O    | 0.88533(22)   | 0.30383(32)   | 0.76990(46)   | 0.972           |
| O2oo | O    | 0.44905(22)   | 0.32661(33)   | 0.42631(45)   | 1.002           |
| O2ox | O    | 0.95575(23)   | 0.34501(33)   | 0.38484(48)   | 1.113           |
| O2ao | O    | 0.20752(22)   | 0.19205(33)   | 0.45295(45)   | 0.964           |
| O2ax | O    | 0.71991(23)   | 0.17949(33)   | 0.47000(47)   | 1.047           |
| O3oo | O    | 0.20522(22)   | 0.44617(31)   | 0.67947(46)   | 0.967           |
| O3ox | O    | 0.70633(22)   | 0.44986(32)   | 0.65457(48)   | 1.007           |
| O3ao | O    | 0.43388(22)   | 0.06725(47)   | 0.60661(47)   | 0.983           |
| O3ax | O    | 0.94845(21)   | 0.04715(31)   | 0.66552(45)   | 0.871           |

Atom labels are chosen to reflect the symmetry-breaking process from the titanite aristotype structure<sup>14</sup>. Numbers in parentheses are estimated standard deviations in the last decimal places quoted. All atoms were refined with anisotropic displacement parameters (not reported here);  $B_{\text{eq}}$  is the equivalent isotropic temperature factor. X-ray diffraction experiments were performed with an Enraf-Nonius CAD4 diffractometer, Mo  $K_\alpha$  radiation, scan mode  $\omega$ , room temperature and pressure. Space group  $I-1$  deduced from the absence condition  $h+k+l=2n+1$  ( $n$  integer), and intensity statistics ( $N(z)$  test and  $E$ -statistics<sup>20,21</sup>) strongly indicated the presence of a centre of symmetry. Structure solution with the SIR program package<sup>22</sup>. Structure refinement with RFINES<sup>23</sup> to  $R_0 = 0.038$ ,  $R_w = 0.036$ ,  $G_{\text{ref}} = 1.7$  for 148 refined parameters and 1,578 reflections observed at  $I > 3\sigma_I$ . Cell parameters from powder diffraction:  $a = 12.9170(2)$  Å,  $b = 8.4504(1)$  Å,  $c = 6.5150(1)$  Å,  $\alpha = 93.161(1)^\circ$ ,  $\beta = 111.475(1)^\circ$ ,  $\gamma = 90.793(1)^\circ$ ,  $V_{\text{cell}} = 660.32(2)$  Å<sup>3</sup>.

The refined structure of  $\text{CaSi}_2\text{O}_5$  has the same basic topology as that of titanite  $\text{CaTiSiO}_5$  (ref. 14; Fig. 1A) from which it can be derived by substitution of the smaller Si cation for the Ti in octahedral coordination, together with mostly small displacements of the atoms and a small distortion of the lattice from monoclinic symmetry. Thus in  $\text{CaSi}_2\text{O}_5$  the calcium remains in a cage site which is coordinated by 7 + 1 (Ax site) or 8 (Ao site) oxygen atoms (Table 2), and there are chains of Si–O polyhedra which run parallel to the  $a$ -axis which are internally bridged by  $\text{SiO}_4$  tetrahedra (Fig. 1b). However, these chains contain three symmetrically distinct silicon sites (denoted Moo, Mox and Ma; Table 2) in place of the single distinct  $\text{TiO}_6$  octahedron in titanite itself. The two silicon sites Moo and Mox in  $\text{CaSi}_2\text{O}_5$  are also octahedrally coordinated, with bond lengths and angles (Table 2, Fig. 2a, b) typical of  $\text{SiO}_6$  octahedra in other high-pressure silicate structures<sup>15</sup>. However, the silicon occupying the Ma site is substantially displaced from its 'ideal' position, and one of the coordinating oxygen atoms, O2ox, is in addition moved to a distance of 2.8305(31) Å from the silicon (Table 2, Fig. 2b). The calculated bond valence<sup>16</sup> for this Si–O distance is 0.04 indicating that there is no significant bonding between the silicon and the O2ox oxygen. This silicon site is therefore coordinated by five oxygens;  $\text{CaSi}_2\text{O}_5$  is the first inorganic crystalline phase proved by structural analysis to contain  $\text{SiO}_5$  groups as an integral part of its structure. The result confirms the above-mentioned allocation of a peak at  $-150$  p.p.m. in the  $^{29}\text{Si}$  MAS–NMR spectra of  $\text{CaSi}_2\text{O}_5$  to pentacoordinate silicon, and the identification of this structure with 'phase Y' of that study<sup>9</sup>.

The geometry of the  $\text{SiO}_5$  group shares some similarities with the pentacoordinate silicon sites found in a variety of organic compounds<sup>17</sup>. In the absence of steric hindrance the common geometry of pentacoordinate silicon in these organic compounds is a trigonal bipyramid, in which the equatorial bonds are shorter than the apical bonds (even when the ligands are the same). The  $\text{SiO}_5$  group in  $\text{CaSi}_2\text{O}_5$  could be thought of having this same

geometry if the O3ao and O3ax oxygens are considered to be the apical ligands. However, this pattern of long Si–O3 bonds and shorter Si–O1 and Si–O2 bonds is also apparent in the two Si octahedra present in the  $\text{CaSi}_2\text{O}_5$  structure. The fact that these both have the same connectivity to other coordination polyhedra as the pentacoordinate silicon site indicates that the bond length pattern in the latter is the result of bonding requirements of the oxygen ligands. In other respects the  $\text{SiO}_5$  group deviates strongly from the geometry found in trigonal bipyramids. Most obviously, the O3ao–Si–O3ax apical bond angle is  $13.6(2)^\circ$  from the  $180^\circ$  required for a regular trigonal bipyramid. In addition, in organic compounds in which the two apical bonds are unequal in length (sometimes as a result of steric effects as here, sometimes because the ligands are not the same) the central silicon atom resides on the side of the equatorial plane towards the closer apical ligand<sup>7</sup>, whereas the Si in  $\text{CaSi}_2\text{O}_5$  lies exactly (within experimental uncertainties) within the plane defined by the equatorial O1o, O1x and O2oo bonds. Last, the equatorial O–Si–O bond angles are very unequal (Fig. 2c) in contrast to the  $120^\circ$  angles in an ideal trigonal bipyramid. We therefore conclude that the geometry of the pentacoordinate silicon in  $\text{CaSi}_2\text{O}_5$  is probably better described as a distorted square-based pyramid, with the O2oo oxygen being the apex and O1o–O3ao–O1x–O3ax forming the base.

Figure 2 clearly shows the structural relaxation that has occurred upon 'removal' of the sixth oxygen, O2ox, from the coordination sphere of the silicon. The oxygen opposite O2ox, O2oo, has moved in towards the silicon, and the basal oxygens have rotated towards the vacated position of the sixth oxygen. This relationship (between the geometry of

TABLE 2 Bond distances and polyhedral parameters in  $\text{CaSi}_2\text{O}_5$ 

| Site | Oxygen               | Distance (Å)          | Site | Oxygen               | Distance (Å)          |
|------|----------------------|-----------------------|------|----------------------|-----------------------|
| Ao   | —O1x                 | 2.304(3)              | Ax   | —O1o                 | 2.350(3)              |
|      | —O2oo                | 2.487(3)              |      | —O2ox                | 2.309(3)              |
|      | —O2ox                | 2.410(3)              |      | —O2ax                | 2.585(3)              |
|      | —O2ao                | 2.460(3)              |      | —O2ax                | 2.897(3)              |
|      | —O3oo                | 2.354(3)              |      | —O3oo                | 2.404(3)              |
|      | —O3ox                | 2.447(3)              |      | —O3ox                | 2.563(3)              |
|      | —O3ao                | 2.500(3)              |      | —O3ax                | 2.448(3)              |
|      | —O3ao                | 2.454(3)              |      | —O3ax                | 2.468(3)              |
| Moo  | Average              | 2.427                 | Mox  | Average              | 2.503                 |
|      | Volume               | $22.92 \text{ \AA}^3$ |      | Volume               | $25.28 \text{ \AA}^3$ |
|      | QE                   | 1.0022                |      | QE                   | 1.0015                |
|      | AV                   | $4.02^\circ$          |      | AV                   | $1.62^\circ$          |
|      | —O1o ( $\times 2$ )  | 1.740(3)              |      | —O1x ( $\times 2$ )  | 1.756(3)              |
|      | —O2ao ( $\times 2$ ) | 1.843(3)              |      | —O2ax ( $\times 2$ ) | 1.786(3)              |
| Ma   | —O3oo ( $\times 2$ ) | 1.792(3)              | Mox  | —O3ox ( $\times 2$ ) | 1.855(3)              |
|      | Average              | 1.792                 |      | Average              | 1.799                 |
|      | Volume               | $7.65 \text{ \AA}^3$  |      | Volume               | $7.75 \text{ \AA}^3$  |
|      | QE                   | 1.0022                |      | QE                   | 1.0015                |
|      | AV                   | $4.02^\circ$          |      | AV                   | $1.62^\circ$          |
|      | —O1o                 | 1.671(3)              |      | —O1x                 | 1.682(3)              |
| Too  | —O2oo                | 1.637(3)              | Ma   | —O2ox                | 2.831(3)              |
|      | —O2ao                | 1.612(3)              |      | —O2ox                | 2.831(3)              |
|      | —O3ox                | 1.639(3)              |      | —O3ax                | 1.824(3)              |
|      | —O3ax                | 1.632(3)              |      | —O3ax                | 1.824(3)              |
|      | Average              | 1.630                 |      | Volume (5)           | $4.03 \text{ \AA}^3$  |
|      | Volume               | $2.20 \text{ \AA}^3$  |      | Volume (5)           | $4.03 \text{ \AA}^3$  |
| Tox  | QE                   | 1.0053                | Tox  | —O2ox                | 1.588(3)              |
|      | AV                   | $20.56^\circ$         |      | —O2ax                | 1.637(3)              |
|      | —O2ox                | 1.637(3)              |      | —O2ax                | 1.637(3)              |
|      | —O2ao                | 1.612(3)              |      | —O3oo                | 1.640(3)              |
|      | —O3ox                | 1.639(3)              |      | —O3oo                | 1.640(3)              |
|      | —O3ax                | 1.632(3)              |      | —O3ao                | 1.627(3)              |
| Tox  | Average              | 1.623                 |      | Average              | 1.623                 |
|      | Volume               | $2.18 \text{ \AA}^3$  |      | Volume               | $2.18 \text{ \AA}^3$  |
|      | QE                   | 1.0037                |      | QE                   | 1.0037                |
|      | AV                   | $13.19^\circ$         |      | AV                   | $13.19^\circ$         |

QE is the quadratic elongation, and AV the angle variance of the polyhedra<sup>24</sup>.

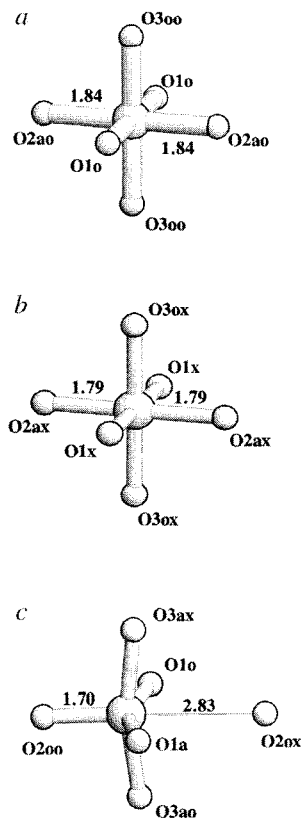


FIG. 2 The coordination of the Moo (a), Moa (b) and Ma (c) sites in  $\text{CaSi}_2\text{O}_5$ . Comparison of c with the Si sites with 6-coordination in a and b indicates the distortion of the Ma site arising from the reduction of the coordination to 5-fold as a result of the removal of the O2ox oxygen. Numbers are interatomic distances in ångströms.

the  $\text{SiO}_5$  group and that of the octahedral groups of  $\text{SiO}_6$  in  $\text{CaSi}_2\text{O}_5$ ) immediately suggests that similar coordination polyhedra for pentacoordinate silicon might be expected to form on the creation of oxygen vacancies in high-pressure silicates whose normal structure contains  $\text{SiO}_6$  octahedra. Such defects are expected to play a central role in oxygen diffusion in the dominant phase of the Earth's lower mantle, (Mg, Fe) $\text{SiO}_3$  perovskite (see, for example, ref. 8). The detailed geometry that we have determined for such a  $\text{SiO}_5$  group will allow more realistic calculations to be performed on the energetics of the formation of such defects and their role in determining diffusion rates in the Earth's lower mantle, as well as their possible role in amorphisation of high-pressure silicates such as  $\text{CaSiO}_3$  perovskite<sup>18</sup> induced by pressure release. The presence of pentacoordinate silicon in glasses and melts has previously been inferred from the observation of signals at approximately  $-150 \text{ p.p.m.}$  in  $^{29}\text{Si}$  MAS-NMR spectra<sup>1,2</sup>, and the observation of a similar signal from  $\text{CaSi}_2\text{O}_5$  (ref. 9) coupled with our structural analysis confirms this assignment. This opens up the possibility of the quantification of pentacoordinate silicon in glasses and its possible influence on network ion diffusivities<sup>4,5</sup> and associated viscosity reduction in silicate melts at high pressures<sup>19</sup>. □

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## The effect of small-scale inhomogeneities on ozone depletion in the Arctic

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THE chemical processes involved in the depletion of polar stratospheric ozone are now fairly well understood<sup>1,2</sup>. But the effect of small-scale stirring and mixing of the chemical species involved can be misrepresented in three-dimensional chemical-transport models because of their coarse resolution. Because of the non-linearities in the chemical rate laws, especially those involving chlorine in the main catalytic cycle, these effects can be important—particularly in the Arctic, where the polar vortex is less uniform and less isolated from surrounding air than in the Antarctic. Here we use a very-high-resolution model with simplified ozone-depletion chemistry to show that the depletion is sensitive to small-scale inhomogeneities in the distribution of reactant species. Under the conditions of the winter of 1994–95 the effect is large enough to account for the observed discrepancies of about 40% between modelled and observed ozone depletion in the Arctic environment<sup>3–5</sup>.

*In situ* observations within the lower stratosphere show unambiguously the presence of sharp horizontal and vertical gradients, and of laminated structure, in the distribution of chemical components which are consistent with the current understanding of advection on isentropic surfaces<sup>6</sup>. Dynamically induced heterogeneities may persist for several weeks. Chemical reactions that depend on mixing of two components, or have nonlinear dependence on concentration, can be strongly affected<sup>7</sup>. State-of-the-art three-dimensional models of atmospheric chemistry can perform detailed modelling of the chemical reactions but are far from resolving the relevant transport and mixing processes<sup>2</sup>. We suggest here that taking into account small-scale inhomogeneities in the distribution of reactant species has a major effect on the predictions of such models.

Because stratospheric motion is thought to be constrained largely within horizontal layers down to scales of 1–10 km where three-dimensional motion is expected to dominate<sup>8,9</sup>, we use here a two-dimensional horizontal transport model on the potential temperature surface  $\theta = 450$  K with a simplified, albeit fairly

realistic, model of the ozone chemistry. Very high resolution is obtained using a two-dimensional finite-volume scheme on the sphere<sup>10</sup> with 1,310,720 triangles and a mesh size  $\delta h = 15$  km ( $\delta h$  being the distance between the centroids of two neighbouring triangles) which is corrected to ensure second-order accuracy, positivity and stability<sup>11</sup>. Because our aim is to estimate the order of magnitude of the effect of horizontal inhomogeneities, we neglect the vertical advection, which may alter the quantitative comparison with observations.

The ozone variation in the model is governed by the catalytic cycle known to be the most effective in the stratosphere<sup>12</sup>, involving the self-reaction of ClO followed by the photolysis of its dimer Cl<sub>2</sub>O<sub>2</sub> (refs 13, 14). Chlorine monoxide and its dimer

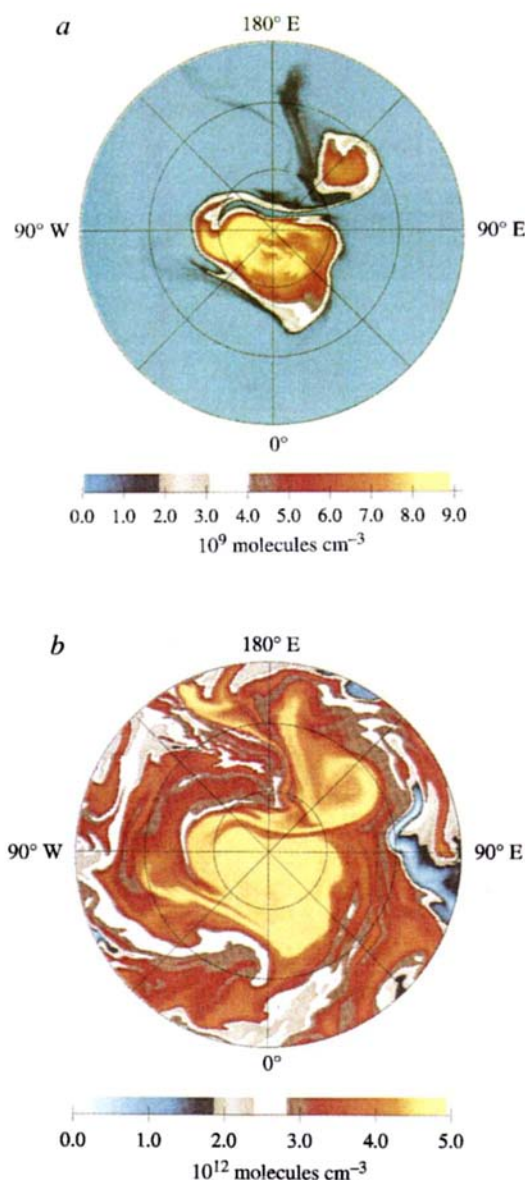


FIG. 1 *a*, Active chlorine concentration and *b*, ozone concentration at 12:00 GMT on 14 February at 450 K in the highest resolution (simulation E15). The projection is stereographic from 25° N to the pole. Latitude circles are at 45° and 70° N. The low level of ozone near 135° E, 60° N, over Siberia, cannot be due to mixing with subtropical air as ClO, would also be diluted. This is a clear indication of depletion, also confirmed by ground-based measurements<sup>21</sup>, which is due to the strong deformation of the vortex expelling activated polar air over mid-latitude regions where photolysis is intense.

are grouped under active chlorine  $\text{ClO}_x$  with  $[\text{ClO}_x] = [\text{ClO}] + 2[\text{Cl}_2\text{O}_2]$ . Ozone and  $\text{ClO}_x$  are the two chemical components transported by the flow. As the reactions following the photolysis of  $\text{Cl}_2\text{O}_2$  are fast, the rate for the whole cycle during daytime is determined by the equilibrium between  $\text{ClO}$  and the dimer. Thereby, the ozone concentration evolves as:

$$\frac{d[\text{O}_3]}{dt} = -2k_{\text{ClO}+\text{ClO}} R[\text{M}][\text{ClO}]^2 \quad (1)$$

where the ambient concentration  $[\text{M}]$  depends on temperature and pressure,  $R$  is given by  $R = J_{\text{Cl}_2\text{O}_2} / (J_{\text{Cl}_2\text{O}_2} + k_{\text{Cl}_2\text{O}_2}[\text{M}])$ ,  $k_{\text{ClO}+\text{ClO}}$  is the reaction of the dimer formation and  $k_{\text{Cl}_2\text{O}_2}$  is the reaction rate of its thermal decomposition<sup>15</sup>. The photodissociation frequencies  $J_{\text{Cl}_2\text{O}_2}$  are interpolated from a pre-calculated table as a function of solar zenith angle. During daylight,  $[\text{ClO}]$  is inferred from  $[\text{ClO}_x]$  using chlorine partitioning and the relation  $(J_{\text{Cl}_2\text{O}_2} + k_{\text{Cl}_2\text{O}_2}[\text{M}])[\text{Cl}_2\text{O}_2] = k_{\text{ClO}+\text{ClO}}[\text{M}][\text{ClO}]^2$ . Polar stratospheric clouds (PSC) are predicted as a function of temperature with no distinction between PSC I and type II water-ice PSCs, or inclusion of sedimentation processes. Rather, PSCs are assumed to form instantaneously at any grid point where  $T < 195 \text{ K}$ . Active chlorine is released at the PSC's surface, at a constant rate  $\alpha = 6 \times 10^4 \text{ molecules cm}^{-3} \text{ s}^{-1}$ , and capped such that the total amount of  $\text{ClO}_x$  is less than 3 parts per billion by volume, p.p.b.v. Chlorine is deactivated through immediate recombination with  $\text{NO}_2$  produced by photodissociation of  $\text{HNO}_3$ , setting a uniform and constant value of gaseous  $\text{HNO}_3$  to 9 p.p.b.v. outside the PSC, reduced to 1 p.p.b.v. at the PSC's surface. Ozone loss is estimated by calculating the difference between the chemical mode (transport + chemistry) and the dynamical mode (transport only).

The data used are winds and temperatures analysed every 6 hours on 14 pressure levels, provided by the European Center for Medium-Range Weather Forecasts (ECMWF) in the horizontal resolution T42 ( $2.8^\circ \times 2.8^\circ$ ), and interpolated onto the  $\theta = 450 \text{ K}$  isentropic surface<sup>16</sup>. Initial distribution of  $\text{O}_3$  is taken from the three-dimensional transport-chemistry model REPROBUS<sup>2</sup> ( $2^\circ \times 2^\circ$ ), on 5 January 1995 at 12:00 GMT. The initial concentration of  $\text{ClO}_x$  is set to  $2.62 \times 10^9 \text{ molecules cm}^{-3}$  ( $\sim 1 \text{ p.p.b.v.}$ ). It is

consistent to use fairly low-resolution wind as the strain is dominated by the largest eddies in layerwise flows<sup>17</sup>. More uncertainty remains on the possible role of small-scale temperature inhomogeneities in the generation of PSCs.

Temperatures in mid-December 1994 to mid-February 1995 were consistently cold, dropping continuously below the conventional threshold value for PSCs formation. By mid-January to mid-February, PSCs were located at the periphery of the polar vortex where strong winds moved substantial amounts of air mass through them, processing a large volume of air into chemically perturbed state. High local values of  $[\text{ClO}]$  build up in the model, up to  $3.8 \times 10^9 \text{ molecules cm}^{-3}$  ( $\sim 1.6 \text{ p.p.b.v.}$ ) on 23 January and  $3.5 \times 10^9 \text{ molecules cm}^{-3}$  ( $\sim 1 \text{ p.p.b.v.}$ ) consistent with observations<sup>18</sup>, owing to the lack of PSCs and deactivation triggered by solar radiation. In early March, a cold spell again enhanced  $[\text{ClO}]$  to the level of early February, dramatically increasing ozone depletion during the month, as activated air was exposed to sunlight (Fig. 2). This tendency has been seen in satellite measurements<sup>18</sup>. At the end of March, the polar vortex was highly distorted by the final warming and strong stirring took place within the vortex but mostly at its periphery.

The sensitivity of ozone depletion to small-scale inhomogeneities in the distribution of reactant species arises from the non-linearity of the governing law (equation (1)). If  $\text{ClO}_x$ , initially released within a finite volume, is diluted by a factor of two, it contaminates a double volume but with half its initial concentration. Therefore, the total ozone destruction by the reaction of equation (1) is reduced by a factor of two with respect to the undiluted case. It is easy to see how crucial is the correct representation of dilution in modelling the ozone chemistry. Using the simulation performed at the highest available resolution  $\delta h = 15 \text{ km}$  as a reference (simulation E15), we have investigated the effect of resolution by reducing  $\delta h$  to 30 km (simulation E30), and by further adding an explicit diffusion term with two different values for the diffusivity,  $\nu_1 = 8 \times 10^4 \text{ m}^2 \text{ s}^{-1}$  (simulation D1) and  $\nu_2 = 3 \times 10^5 \text{ m}^2 \text{ s}^{-1}$  (simulation D2) which provide effective horizontal resolution of  $\sim 100$  and  $\sim 200 \text{ km}$  (based on a strain value of  $10^{-5} \text{ s}^{-1}$ ), within the range of the REPROBUS model.

The effect is indeed considerable (Fig. 3): maximum ozone depletion rises to 64.5% in E15 but drops to 53% in E30, and to 39.5% and 21.6% in D1 and D2. Main differences lie within the polar vortex where maintaining large values of  $[\text{ClO}_x]$  over February and March enhances ozone depletion. Maximum depletion is located over filamentary structures inside the vortex, which replicate that of  $[\text{ClO}_x]$ , and are missed at lower resolution. The high-resolution calculation also predicts isolated ozone destruction of  $\sim 15\%$  with peak values at 34.5% over the Eastern Pacific, near  $30^\circ \text{ N}$  which is consistent with lidar observations reporting ozone depletion at middle latitudes<sup>19</sup>. This destruction can be traced back to the advection of a long and thin filament expelled from the polar vortex and carrying air rich in  $\text{ClO}_x$  towards middle latitudes where it reacts with  $\text{O}_3$ . This effect is weakened when the resolution is degraded and disappears in the most diffusive case (Fig. 3b–d).

The predicted averaged ozone loss within the vortex (Fig. 4a) ranges from 17% to 43% at the end of March. The comparison with SAOZ spectrometer measurements<sup>22</sup> measurements and the results of REPROBUS is made under the assumption that the variations in ozone column are mainly weighted by the contribution near 450 K. Our model clearly fails in reproducing the initial ozone loss in January. This discrepancy could be due to the absence of bromine which may account for 30–40% of the ozone loss in this period<sup>20</sup>. In addition, our model does not take into account the diabatic descent, nor the generation of ozone by photolysis of  $\text{O}_2$ , which may lower ozone loss in March<sup>19</sup>. Nevertheless, the main point arising from this comparison is that the discrepancy by a factor of 1.4 by the end of March between the data and the prediction of REPROBUS is very close to the predicted effect of resolution in our model. This result is not limited by the simplicity of our model and should apply under

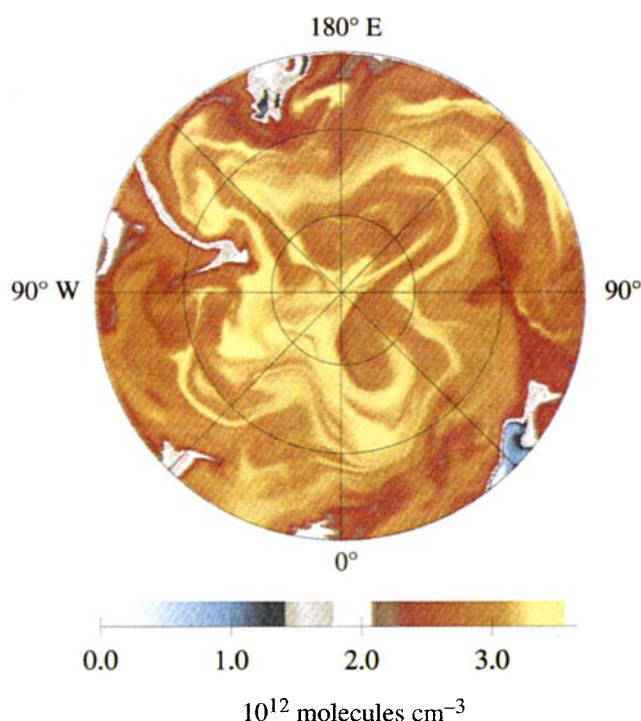


FIG. 2 Concentration of ozone at 12:00 GMT on 21 March at 450 K for the E15 simulation.



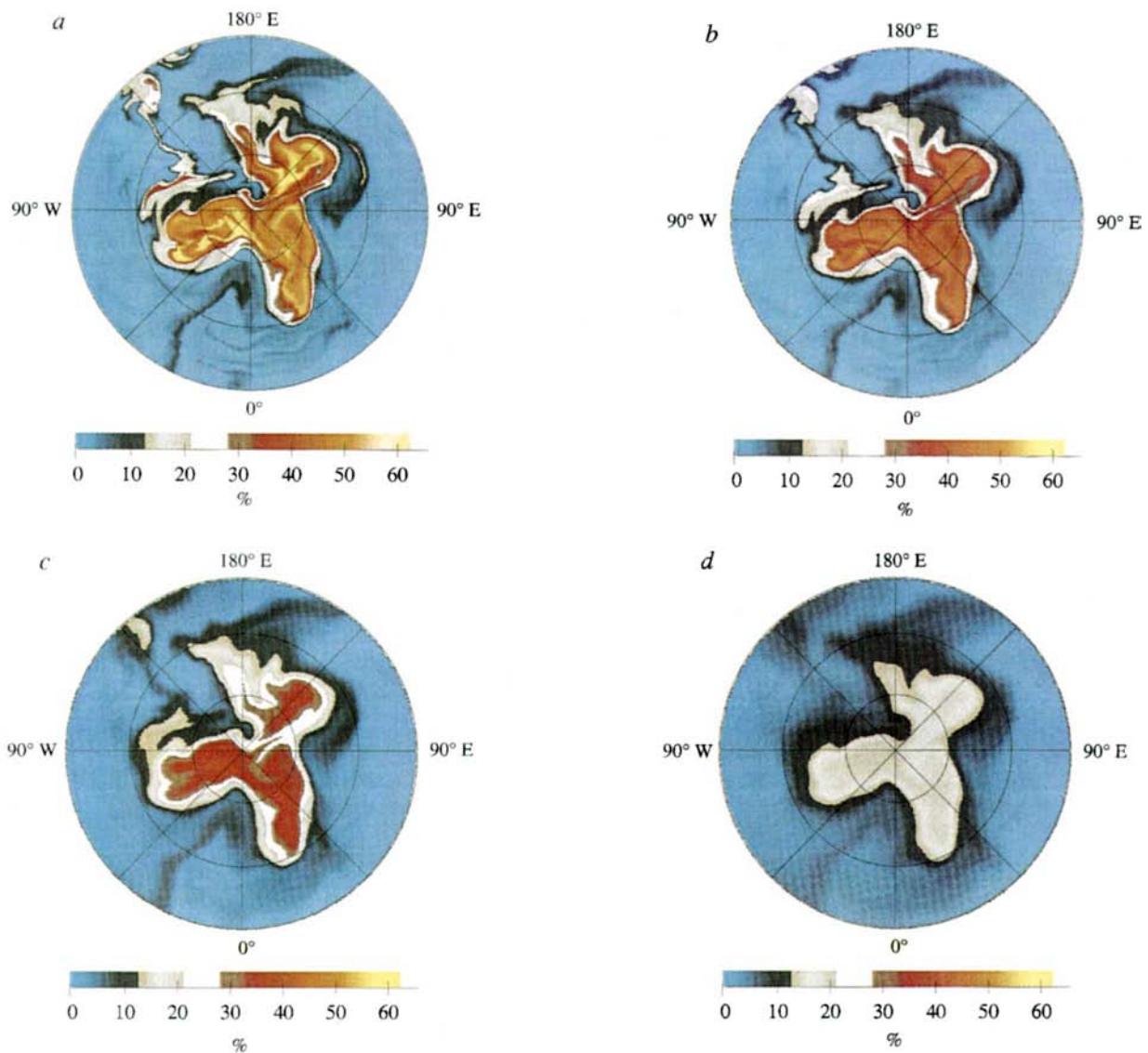


FIG. 3 Ozone loss (%) for the E15 simulation (a), the E30 simulation (b), the D1 simulation (c) and the D2 simulation (d), at 12:00 GMT on 30 March at 450 K.

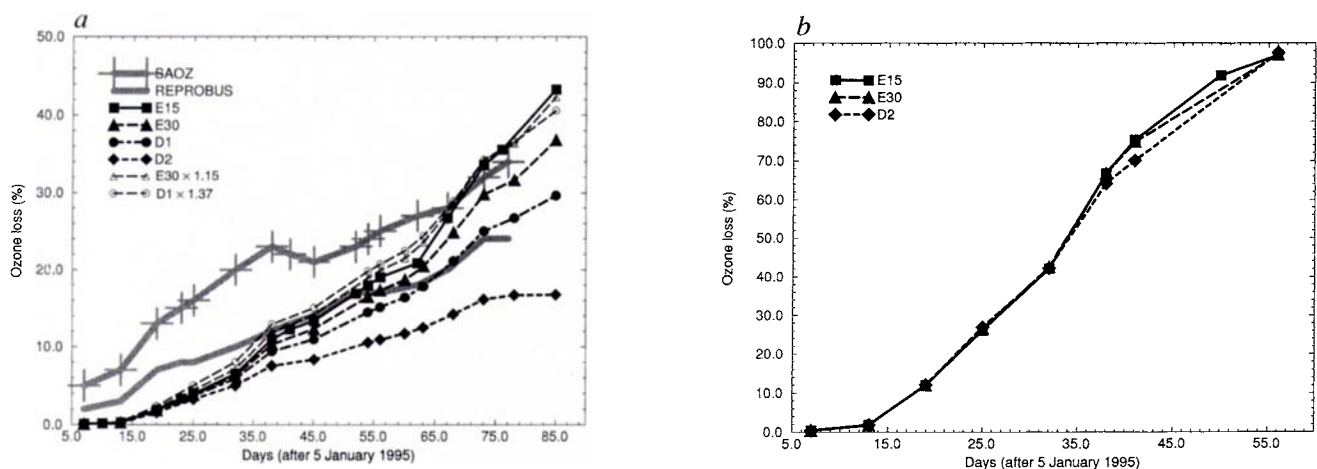


FIG. 4 a, Ozone loss (%) within the vortex as a function of time, at 450 K, from 5 January to 30 March, for the E15 simulation (solid line, squares), the E30 simulation (long dashed line, triangles), the D1 simulation (dot-dashed line, circles) and the D2 simulation (dashed line, diamonds). SAOZ measurements (grey line, crosses) and REPROBUS results (grey line, no plotting symbols) are adapted from refs 3 and 4. b, Ozone loss (%) within

the vortex as a function of time under Antarctic conditions (increased  $\alpha$ ), from 5 January to 1 March, for the E15 simulation (solid line, squares), the E30 simulation (long dashed line, triangles) and the D2 simulation (dashed line, diamonds). The boundary of the polar vortex at 450 K is defined by the contour where potential vorticity is  $25 \times 10^{-6} \text{ K m}^2 \text{ kg}^{-1} \text{ s}^{-1}$ .



more realistic conditions as long as ozone depletion by chlorine dominates in the Arctic.

It is important to note that ozone is only partially destroyed. In the Antarctic, where denitrification makes available a large amount of chlorine, almost complete destruction of ozone is observed. One would expect that the dilution effect here may not be as important as in the Arctic. We tested this by artificially increasing  $\alpha$  by a factor of 10 in our calculation to imitate the conditions prevailing in the Antarctic. Much higher depletion is then observed (Fig. 4b) but there is almost no sensitivity to dilution. This is not to say that mixing effects are absent. The relevant mechanism, not considered here, is the sensitivity of chlorine deactivation to the mixing of denitrified polar air with subtropical air<sup>8</sup> for which higher diffusivity or the lack of numerical resolution enhances the chemical reactivity.

We investigated whether the effect of small-scale inhomogeneities could be parametrized in low-resolution three-dimensional chemical models. A good fit to the curve of total ozone depletion for simulation E15 can be obtained by multiplying the corresponding curves for E30 and D1 respectively by a factor of 1.15 and by a factor of 1.37 (dashed curve with empty symbols in Fig. 4a) which is equivalent to increasing  $k_{\text{ClO}+\text{ClO}}$  in equation (1). In the case of chlorine deactivation, where chemical compounds are initially separated, Thuburn and Tan<sup>8</sup> propose decreasing the reaction rate in the model. The influence of dynamics on chemistry is complex so that designing robust parametrizations of small-scale

inhomogeneities might not prove an easy task. Meanwhile modellers should be aware that their results depend on the spatial resolution and that ultra-high-resolution modelling might be necessary to reach accurate results. □

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## Possible role of dust-induced regional warming in abrupt climate change during the last glacial period

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RECORDS from loess, sediments and ice cores indicate that the concentrations of tropospheric aerosols were higher in glacial periods than they are today, and that they peaked just before glacial terminations<sup>1–10</sup>. Energy-balance models have suggested<sup>11–14</sup> that these high glacial aerosol loadings were a source of glacial cooling of the order of 1–3 °C. Here we present a different view based on three-dimensional climate simulations, which suggest that high glacial dust loading may have caused significant, episodic regional warming of over 5 °C downwind of major Asian and ice-margin dust sources. Less warming was likely close to and over the oceans because of local cooling by sea-salt and marine sulphate aerosols. Abrupt changes in dust loading are associated with the Dansgaard–Oeschger and Heinrich climate events and with glacial termination<sup>3,8,15</sup>, suggesting that dust-induced warming may have played a role in triggering these large shifts in Pleistocene climate.

To examine the potential role of tropospheric dust in glacial climates, we carried out two sets of simulations with the NASA Goddard Institute for Space Studies (GISS) general circulation

model (Model II, using 4° × 5° latitude–longitude resolution, full seasonal cycle, interactive land surface and clouds<sup>16,17</sup>). First, two five-year simulations (with fixed sea surface temperature, SST) were run with identical late glacial conditions<sup>18</sup> but with different atmospheric dust loadings. In the ‘modern-dust’ simulation, we used present-day monthly mean dust<sup>19</sup> as shown in Fig. 1a, b). In the ‘glacial-dust’ simulation, average modern-day desert silt and clay mineral dust<sup>19,20</sup> was prescribed with peak abundance in the lower troposphere over each 4° × 5° model grid box containing some fraction of the North American ice sheet or desert (Fig. 1c). The optical depth of glacial dust was specified to be 0.168, in accordance with ice-core observations<sup>1,7</sup>, distributed uniformly over the affected grid boxes.

Comparing ‘glacial-dust’ with ‘modern-dust’ simulations shows warming patterns (Fig. 2a) that differ substantially from the pattern of applied radiative forcing (Fig. 1c); this points to the importance of regional differences in feedback interactions that arise from changes in static stability due to solar heating and longwave heating/cooling by dust aerosols<sup>19,20</sup>. This behaviour differs from the classical response inferred from changes in energy balance arising from aerosol effects on planetary albedo<sup>21,22</sup>. Except for regions of northern Canada and Alaska, the dust-induced average annual warming was greater at progressively higher latitudes, and was greatest (up to 4.4 °C) in regions with dust over high-albedo snow- and ice-covered areas. Because the SSTs were fixed in these simulations, the global temperature change was small. Contributing changes in cloud cover, atmospheric water vapour, and surface albedo are listed in Table 1.

Our results are consistent with recent assessments of how mineral dust affects present climate<sup>19,20</sup>. Decreased backscattering of incident solar radiation occurs when the aerosol overlies high-albedo snow- or ice-covered surfaces, whereas solar backscattering is increased when the dust overlies dark land and ocean surfaces. The effective cloud single scattering albedo decreases when dust occurs within clouds<sup>19</sup>. In addition to its shortwave effect, dust loading also contributes a small, but significant, amount of greenhouse warming through increased absorption of thermal radiation<sup>17</sup>. Within this general context, specific causes of surface warming varied from region to region. Typically, regions with reduced planetary albedo are associated with increased solar

heating of the troposphere and surface. Some of the warming resulted from tropospheric trapping of thermal radiation. In addition, changes in atmospheric pressure and circulation patterns were evident, indicating that some of the warming arose from warm air being advected from the south and from warmer ocean areas.

These simulations with fixed SSTs were intended to focus on the regional sensitivity of the direct radiative effects of mineral dust aerosols. The overall global response was limited by the fixed SSTs, but the negative net radiation at the top of the atmosphere (Table 1) implies global radiative disequilibrium such that the globe would cool if the SSTs were allowed to adjust.

A more complete test of model sensitivity to mineral dust is provided by our second set of GCM simulations which are identical to the first set but with interactive SSTs. These simulations were run to full equilibrium (75 years) using ocean heat transports calculated from the fixed-SST 'modern-dust' simulation. Here, North Atlantic heat transports implied in the fixed-SST 'glacial-dust' simulation were not significantly different from today's, although to have maintained the Pacific and global SSTs under late glacial conditions would have required increasing peak transports by 18 and 6%, respectively.

Surface air temperatures obtained with interactive SSTs are only slightly cooler than those with fixed SSTs, with differences occurring primarily over land. Several of the feedbacks changed sign and thus mitigated the temperature changes. In particular, low cloud cover decreased, and the planetary albedo change became negative. Overall, the more realistic interactive-SST ocean reduced the amount of mid- to high-latitude warming from 4.4 °C (Fig. 2a) to 2.4 °C (Fig. 2b), but the principal regions of significant warming remain concentrated in areas where the dust loading coincides with snow- or ice-covered land surfaces.

Some aspects of our simulations may have underestimated the full potential of regional dust-induced warming. First, although

TABLE 1 Regional annual-average global climate anomalies

|                                      | Fixed SSTs | Interactive SSTs |
|--------------------------------------|------------|------------------|
| Surface air temperature (°C)         | 0.19       | 0.13             |
| Peak warming over land (°C)          | 4.40       | 2.40             |
| Net radiation at top of atmosphere*† | -0.40      | 0.00             |
| Planetary albedo†                    | 0.09       | -0.08            |
| Ground albedo†                       | -0.32      | -0.09            |
| Atmospheric water vapour (mm)        | 0.30       | 0.10             |
| Total cloud cover (%)                | 0.40       | 0.00             |
| High cloud (%)                       | -0.10      | 0.10             |
| Low cloud (%)                        | 0.70       | -0.20            |

These anomalies are for our 'glacial-dust' minus 'modern-dust' simulations (see text).

\* Negative value implies atmospheric disequilibrium.

† All radiation terms in  $\text{W m}^{-2}$ .

the magnitude of our dust loading was similar to Greenland ice-core estimates, these ice cores were far from hypothesized Asian desert and North American ice-margin dust sources<sup>1,7,23,24</sup>. True loadings were probably much higher closer to these source regions. Second, modern studies highlight the regional and episodic nature of major aerosol loadings<sup>25,26</sup>, and make it clear that dust-induced climate changes could have been regionally much more intense than those implied by our simulations with modest levels of uniform optical depth. Also, we did not include the reduction of snow albedo by dust, an unknown but potentially important factor which would serve to amplify the warming. Last, we did not include microphysical aerosol-cloud interactions that could have altered cloud extent and cloud optical properties<sup>27,28</sup>.

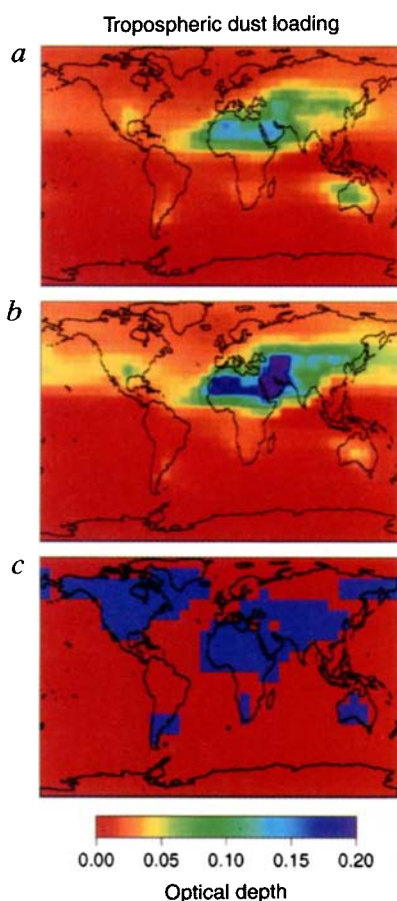


FIG. 1 Dust loading prescribed in the climate model experiments. Different monthly modern dust loadings (a, annual average; b, June average) were prescribed using observed optical depths<sup>17,19</sup>; in contrast the same dust loading was prescribed for each month of the glacial simulations (c). Prescribed regional glacial dust loadings in the Northern Hemisphere are in accordance with time-averaged observations<sup>1,7</sup>; it is likely that episodic peak glacial loadings may have been significantly greater than those we used.

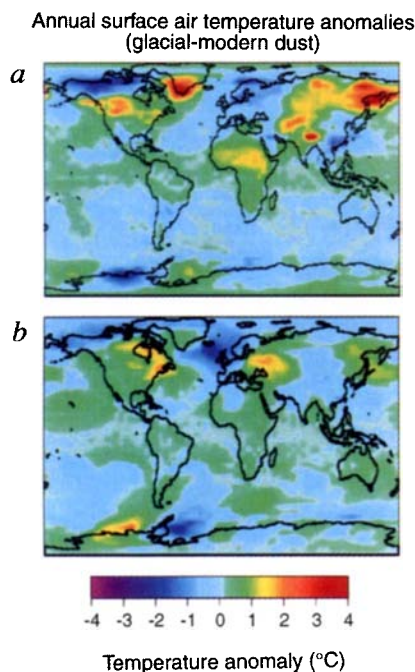


FIG. 2 Simulated annual surface air temperature anomalies between the 'glacial-dust' and 'modern-dust' simulations. Two sets of experiments were carried out, one with fixed sea surface temperatures (a), and the other with interactive sea surface temperatures (b). Both sets show significant warming in areas of glacial tropospheric dust loading. Peak dust loading, and hence warming, was probably significantly greater than shown here (see text).

Here we have simulated the effects of dust aerosol only. For a more complete study, other aerosols would need to be considered<sup>12,26</sup>. In particular, tropospheric sea-salt concentrations were as high as one-third of the total dust loading in some marine and coastal locations<sup>7,8</sup>. But rapid fall-off of sea-salt concentration inland from ocean sources due to the typically large size of salt particles make it less likely that sea salt could have achieved continental distributions nearly as significant as the mineral dust<sup>29</sup>. Also, glacial marine sulphate loadings were significantly higher in Southern Hemisphere polar regions compared to present, but smaller in the Northern Hemisphere, unlike the situation with dust<sup>8,30</sup>. Carbon/soot concentrations also appear to have been negligible during glacial periods<sup>31</sup>. Thus, mineral dust appears to have been the most globally distributed aerosol, with the largest

radiative effect over snow- and ice-covered regions.

Tropospheric dust loading would be expected to increase steadily with the extent of glaciation. Thus, winds, dust sources, and hence dust entrainment would have been largest along the margins of ice sheets<sup>14</sup>. Peak Asian dust (loess) loading also coincided with the strong winds and minimal monsoon precipitation associated with glaciation<sup>15,32</sup>. These are the same areas where dust would have produced significant warming once the dust sources were sufficiently developed. Greenland ice-core data suggest that dust loading, mostly of Asian origin<sup>23</sup>, peaked just before the well-known Dansgaard-Oeschger warm events<sup>3</sup>, before and during Heinrich events<sup>8</sup>, and just before glacial terminations<sup>2,3,5,7</sup>.

Most recent investigations of these abrupt Pleistocene events, including those that have coupled ice-core events to major shifts in North Atlantic SSTs and regional circulation, indicate that large-scale forcing is needed to explain the simultaneous effect on several different ice sheets<sup>33</sup>. Episodic dust loading may have provided the warming needed to trigger the ice, ocean and atmospheric changes associated with these events. Dust loading and associated snow darkening are leading mechanisms for explaining the abrupt termination of the 100-kyr glacial cycle<sup>34</sup>. The initiation of Heinrich events required some climatic or climate-induced sea-level trigger (for example, dust-induced warming) to decouple ice sheets from their beds and generate the moderate (Dansgaard-Oeschger) or massive (Heinrich) meltwater/iceberg discharges from several different Northern Hemisphere ice sheets<sup>33,35</sup>.

Warming following these events would have led to the formation of extensive lakes which would have trapped dust-sized sediment entrained in outwash<sup>14</sup>. At the same time, hemispheric warming following the Heinrich events<sup>15</sup> would have reduced Tibetan plateau snow cover, increased heating of land in summer, strengthened the Asian monsoon, and stabilized dust sources with higher precipitation<sup>36</sup>. Thus, peak dust loading may have triggered the same climate changes that then led to observed abrupt decreases in dust loading<sup>3,7,8,15</sup>.

Our results point to dust aerosols as a potential source of episodic warming during the last glacial period, and suggest that this warming might be the trigger mechanism needed to account for previously unexplained major abrupt climate events. Our study also highlights the complicated nature of modelling aerosol-induced climate change, and the need for three-dimensional general circulation model simulations to model all aspects of the climate response, particularly at high latitudes with absorbing aerosols and under cloudy sky conditions. New ice-core and geological data are needed to improve our understanding of aerosol characteristics, their sources and transport processes during the Pleistocene. Only then will it be possible to extend our modelling approach to refine the exact magnitude of past dust-induced warming. □

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# Relatively recent construction of the Tien Shan inferred from GPS measurements of present-day crustal deformation rates

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**THE Tien Shan—a high, seismically active intracontinental mountain belt, 1,000–2,000 km north of the Himalaya—has grown as a result of India's collision with Asia<sup>1</sup>. The crustal shortening ( $\sim 200 \pm 50$  km; refs 2, 3) and thickening that gave rise to the Tien Shan accommodates only a small fraction of India's total penetration into Asia (2,000–3,000 km), and the temporal relationship of deformation in this belt to the India–Asia collision remains unclear. Here we report geodetic measurements of the Tien Shan, using the Global Positioning System (GPS), that indicate that the current crustal shortening rate is nearly half of India's convergence rate with Eurasia in this area<sup>4</sup>. We infer a total shortening rate for the Tien Shan of  $\sim 20$  mm yr<sup>-1</sup>, which is approximately twice that inferred previously from the extrapolation of slip rates in the Holocene<sup>5</sup> and earthquake-induced displacements during this century<sup>6</sup>, suggesting that the rate of mountain building in this region has accelerated several-fold since the onset of collision  $\sim 50$ – $55$  Myr ago<sup>6,7</sup>. If, as we argue, the current shortening rate can be extrapolated to geological timescales, then our results suggest that most of the Tien Shan has been constructed during the past 10 Myr, perhaps in response to an increased horizontal force following an abrupt rise of the Tibetan plateau<sup>8,9</sup>.**

The Tien Shan, some 2,500 km long and 300–500 km wide, forms a typical intracontinental mountain belt, far from either the locus of collision between continents or a subduction zone (Fig. 1), although it is a consequence of the collision between India and the rest of Eurasia. Within the belt, thrust or reverse faults, without a consistent vergence, separate easterly trending ranges and basins, some of which contain 2 km or more of late Cenozoic sediments<sup>10–14</sup>. Fault plane solutions of earthquakes<sup>15–19</sup>, surface ruptures of two major earthquakes<sup>20–22</sup>, and widespread Holocene faulting and folding<sup>10,11,13,16</sup> attest to continued thrust faulting and

crustal shortening throughout the Tien Shan.

Although strain within the Tien Shan accommodates India's penetration into the rest of Eurasia, the temporal relationship of deformation to the collision is not clear. Intense Palaeozoic deformation occurred in the area that includes the present Tien Shan<sup>23</sup>, and transport directions of water flow inferred from Mesozoic fluvial sediment adjacent to the present mountain belt imply early and middle Mesozoic relief<sup>24</sup>. Yet the present high topography appears to be Cenozoic in age; early Cenozoic (and rare late Cretaceous) sedimentary strata conformably overlie flat, but tilted, erosional surfaces on individual ranges and collectively define a 'pre-orogenic surface' with little early Cenozoic relief<sup>10–12</sup>. Variations in thicknesses of late Oligocene and early Miocene sediment suggest the subsequent development of relief, a result consistent with cooling ages of rock exposed in the eastern Tien Shan<sup>25,26</sup>. A progressive thickening of the late Cenozoic sedimentary section in basins within the Tien Shan, led several workers to deduce that deformation accelerated in late Cenozoic time<sup>10–13,27</sup>. Because of uncertainties in dating continental deposits and large changes in the geological timescale since much of this work was done, however, it has been impossible to quantify such an inference of acceleration. For instance, Avouac *et al.*<sup>3</sup> extrapolated their estimated rate of Holocene shortening across part of the Tien Shan to infer an initiation of deformation at  $\sim 20$  (+30/–10) Myr, but the large uncertainties of both the Holocene rate and the total shortening prohibit placing tight constraints on the time of initiation of mountain building in the belt.

The relief, distribution of active faults, and seismicity indicate that deformation has been distributed across the Tien Shan (Fig. 1). This contrasts with subduction zones, where a localized thrust zone marks the boundary between two plates, and with collision zones, where one continent has plunged beneath another along a major thrust fault, as has occurred in the development of the Alps<sup>28</sup> or Himalayas<sup>29</sup>. In these respects, the Tien Shan typifies intracontinental mountain belts, like the Nan Shan, Altay, or Gobi-Altay in Asia<sup>1,16</sup>, the Atlas Mountains of North Africa, the Sierras Pampeanas of the Argentine Andes<sup>30</sup>, the Laramide Rocky Mountains of the western United States<sup>17</sup>, and perhaps the Tibetan plateau in its initial stages of Cenozoic development. In the segment of the Tien Shan shown in Fig. 1, a typical fold-and-thrust belt is underscored by its association with (1) the marked flexure of the Tarim Basin, which requires a significant load<sup>31</sup>; (2) the highest rates of current seismicity in the Tien Shan; and (3) the highest range in this segment of the Tien Shan.

To measure the present-day kinematics of deformation, we established an 86-station GPS network in the Republics of Kyrgyzstan and Kazakhstan (Fig. 1). The network shares sites with a regional-scale network established by a collaboration of German scientists from the GeoForschungsZentrum and local scientists, including some of us<sup>32</sup>. Here we rely on measurements from a small part of the 86-site network made in 1992, but also, and more importantly, on measurements made in the central and eastern parts of the network in 1993, in 1994, and twice in 1995 (Fig. 1).

We processed the data in three stages<sup>33</sup>. In the first, we used carrier phases to estimate three-dimensional (3D) coordinates of all sites, parameters representing the satellites' orbits, and other miscellaneous parameters needed to model GPS phase data. For each day of data collection, we processed recordings from a global network of 27–40 tracking sites and, separately, a regional network with two to three global sites included. Until the second campaign in 1995, all global sites lay well outside the Tien Shan. For that campaign, we included one of the newly installed continuously operating stations in the Tien Shan (POL2) in the analysis of global stations. The GPS biases were resolved to integer values for the regional stations but not in the global analyses<sup>33</sup>.

In the second stage, we combined 3D station coordinate estimates and satellite orbit parameters, with their full covariance matrices, and used a Kalman filter estimator<sup>34</sup> to obtain optimum

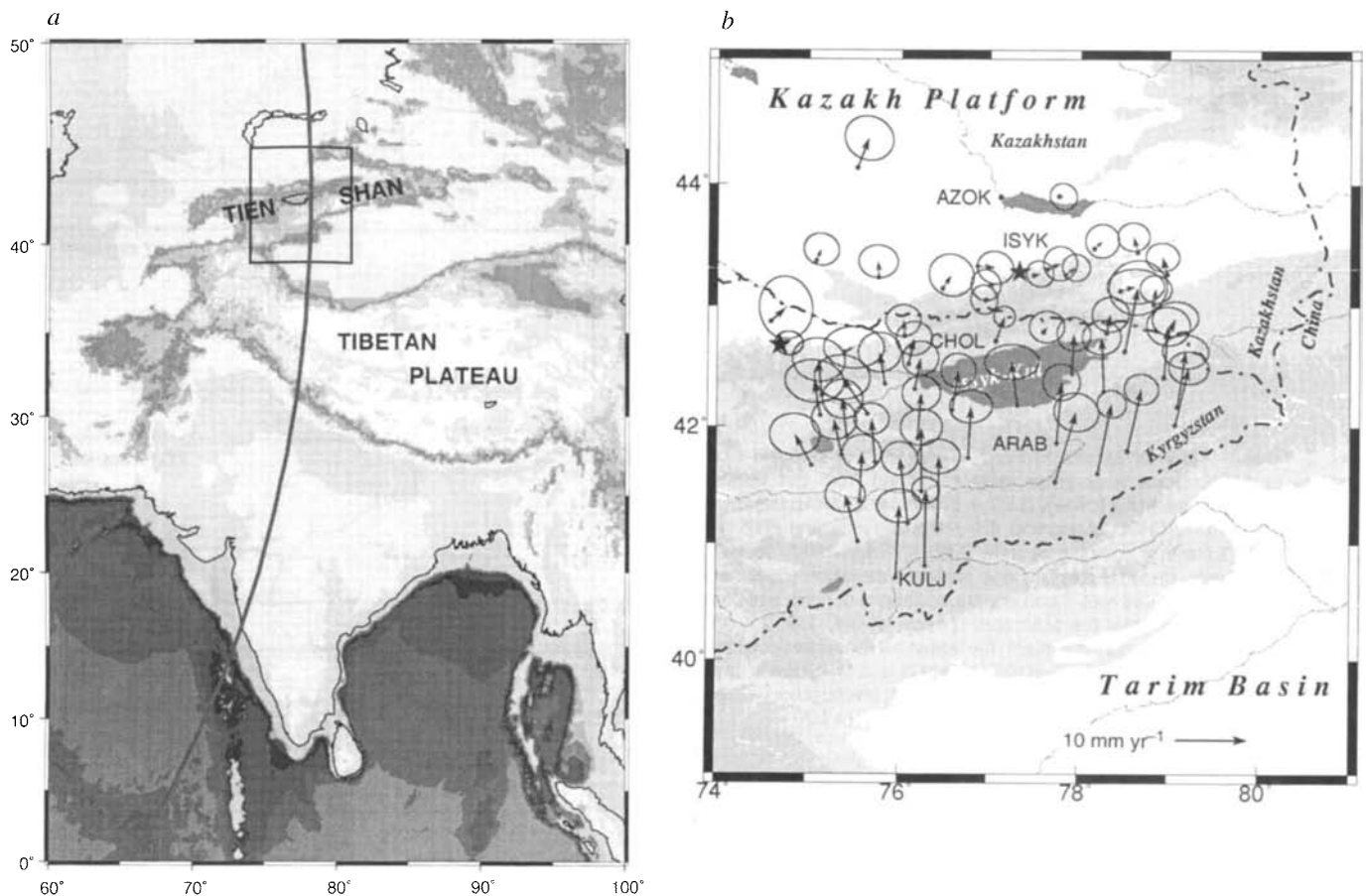


FIG. 1 Maps showing *a*, the study area in relation to present-day topography of central Asia, and *b*, the eastern portion of the network of Global Positioning System sites in the Tien Shan with their measured velocities. In *a*, the arcuate line defines a small circle about the angular velocity vector for relative motion between the Indian and Eurasian plates. The box outlines the region shown in *b*, in which GPS sites are shown by

black dots, with arrows emanating from them to show velocities with respect to AZOK, near the northern edge of the network. Ellipses define 95% confidence regions. Black stars show locations of continuously recording GPS receivers. Our network extends west of that region, but we confine discussion to the results from the area shown here.

estimates of the 3D site coordinates for each campaign. To avoid singularities in this analysis, we constrained all site coordinates weakly, to be within 100 m of estimated coordinates, after which satellite orbit parameters are no longer needed.

In the third stage, we did two types of analysis. In one, we combined estimates of the 3D site positions from each campaign to estimate simultaneously the 3D site coordinates and velocities, with weak constraints on both:  $<100$  m and  $<1$   $\text{mm yr}^{-1}$ , respectively. To this solution, we applied generalized constraints to the rigid-body rotation and translation of its coordinate system to minimize the differences in the horizontal velocities between the observed and previously determined velocities at 13 global sites. This analysis formed our velocity solution, presented with respect to AZOK on the Kazakh Platform (Fig. 1). In a second type of analysis, we considered campaign solutions separately with the coordinates of the 13 global sites constrained to lie within a few millimetres of their estimated positions for each campaign epoch. For this analysis, we generated time-series plots for the coordinates of the regional sites (Fig. 2).

The observed velocity field (Fig. 1) documents strain across the belt from the Kazakh Platform, north of the belt, to the segment near the border with China. Rates range from near zero, in the north, to a maximum at the southern edge of the network of  $13 \pm 2$   $\text{mm yr}^{-1}$  (Figs 2 and 3), and directions are essentially parallel to the convergence between the Indian and Eurasian plates<sup>4</sup>. Because our estimated rate at which AZOK moves with respect to Eurasia— $1.7 \pm 3$   $\text{mm yr}^{-1}$  towards  $\text{N}10^\circ \text{W}$ —is not resolvable, we conclude that only minor plate convergence is

absorbed north of AZOK. Our data do not indicate areas of localized high or low strain rates, except on the eastern profile (Fig. 3), although the large uncertainties in rates cast some doubt on the magnitudes of strain rates there. Within the interior of the belt, especially along the profile in the  $76\text{--}77^\circ$  band, the uniform variation in north–south speed across the Tien Shan implies a roughly homogeneous strain field. The small variation in northward speeds across the network (Fig. 3) suggests that rates across individual faults within the Tien Shan do not exceed a few  $\text{mm yr}^{-1}$ . Thus, the distribution of strain accumulation concurs with the geological and seismological observations of active deformation throughout the Tien Shan<sup>10–13,15–22</sup>.

Because our network spans only  $\sim 70\%$  of the width of the Tien Shan, as measured from the Kazakh Platform across the belt to the Tarim Basin,  $13 \pm 2$   $\text{mm yr}^{-1}$  underestimates the total convergence rate across the belt. A simple extrapolation implies a total convergence rate of  $\sim 20$   $\text{mm yr}^{-1}$  between the Tarim Basin and the Kazakh Platform. Such an extrapolation seems reasonable, given both the nearly constant strain rate that we measure (Fig. 3) and the particularly high terrain, high seismicity and marked flexure of the Tarim lithosphere south of our network. In fact, geological mapping of a fold-and-thrust belt at the southern edge of the Tien Shan  $\sim 500$  km east of our network suggests an average Quaternary shortening rate across it of between 4 and 14  $\text{mm yr}^{-1}$  (B. C. Burchfiel *et al.*, manuscript in preparation), consistent with such an extrapolation. This rapid convergence across the Tien Shan supports the contention that crustal shortening and thickening built the present Tien Shan, with thermal processes associated

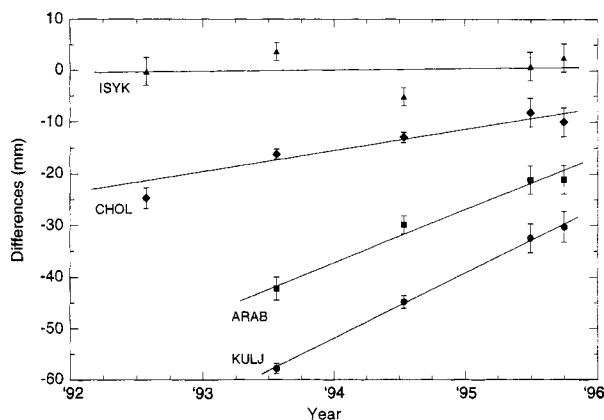


FIG. 2 Time evolution of estimated North coordinates of selected sites relative to AZOK. Black lines show rates estimated from the velocity analysis for four sites, KULJ (circles)  $12.7 \pm 1.0 \text{ mm yr}^{-1}$ , ARAB (squares)  $10.3 \pm 1.0 \text{ mm yr}^{-1}$ , CHOL (diamonds)  $4.0 \pm 0.8 \text{ mm yr}^{-1}$ , and ISYK (triangles)  $0.3 \pm 1.0 \text{ mm yr}^{-1}$ , which lie 349, 232, 131 and 76 km south of AZOK, respectively. Uncertainties are one standard deviation (68% confidence interval). Weighted root-mean-square deviations of north positions from those calculated using the estimated speeds, of 0.4, 1.9, 1.7 and 4.6 mm, respectively, are consistent with the statistical model for ARAB and CHOL. For KULJ, the scatter is 30% of that expected, and for ISYK it is twice that expected. The additional scatter at ISYK is under investigation. Larger errors bars for 1995 reflect fewer days of recording than in 1994.

with asthenospheric upwelling<sup>35</sup> playing at most a minor role.

The shortening rate of  $\sim 20 \text{ mm yr}^{-1}$  across the Tien Shan exceeds previous estimates<sup>3,5</sup> by 50–100%. Seismic moments of earthquakes suggest shortening at an average rate of  $10 (\pm 3) \text{ mm yr}^{-1}$  in this century (ref. 5, and our unpublished work). The high rate of convergence therefore implies either a significant proportion of aseismic strain in the Tien Shan or a deficit in the seismic slip this century, which might indicate a heightened risk of a major earthquake occurring somewhere in the Tien Shan. The apparently uniform strain accumulation across the Tien Shan implies that the risk of such an event is not restricted to the northern or southern margins of the belt, but could occur on virtually any of the major thrust faults within the belt.

The shortening rate of  $13 \pm 2 \text{ mm yr}^{-1}$  strictly applies for only  $\sim 2$  yr. Observations from elsewhere, however, show agreement between rates estimated geodetically for distances sufficiently long that elastic strain is minor and average rates for geological timescales. For instance, geodetically determined slip rates for the San Andreas fault<sup>33</sup> and Sieh and Jahns's<sup>36</sup> average Holocene rate differ by less than the uncertainties of a few  $\text{mm yr}^{-1}$  in each. Similarly, determinations of plate motions from magnetic anomalies since 2 Myr differ by a few  $\text{mm yr}^{-1}$ , at most from those estimated using space geodetic techniques for 5–10 yr (ref. 37). Moreover, on a regional scale, the convergence rate between India and Eurasia has varied insignificantly, by no more than a few  $\text{mm yr}^{-1}$ , since their collision<sup>1,9</sup>. Nevertheless, because we cannot eliminate the possibility that large, essentially rigid blocks, such as that beneath the Tarim Basin, move erratically and not steadily with respect to their neighbours, the equating of a 2-year average rate with a several million year average carries a risk that cannot be evaluated at present. We ignore such a possibility here.

The observed shortening across the western Tien Shan at  $\sim 20 \text{ mm yr}^{-1}$  may be used to estimate the timescale for Cenozoic crustal shortening in the Tien Shan. Assuming that thick crust compensates for the high elevations of the Tien Shan, and that Airy isostasy holds at least approximately, Ulomov<sup>2</sup> and Avouac *et al.*<sup>3</sup> estimated 200 ( $\pm 50$ ) km of Cenozoic shortening. At a roughly constant shortening rate of  $20 \text{ mm yr}^{-1}$ , the entire belt could have been built in  $\sim 10$  Myr. If deformation began as early as 20–30 Myr, it must have accelerated by several times since that time, as Goryachev<sup>10</sup> and Makarov<sup>11</sup> suggested. If a hot uppermost

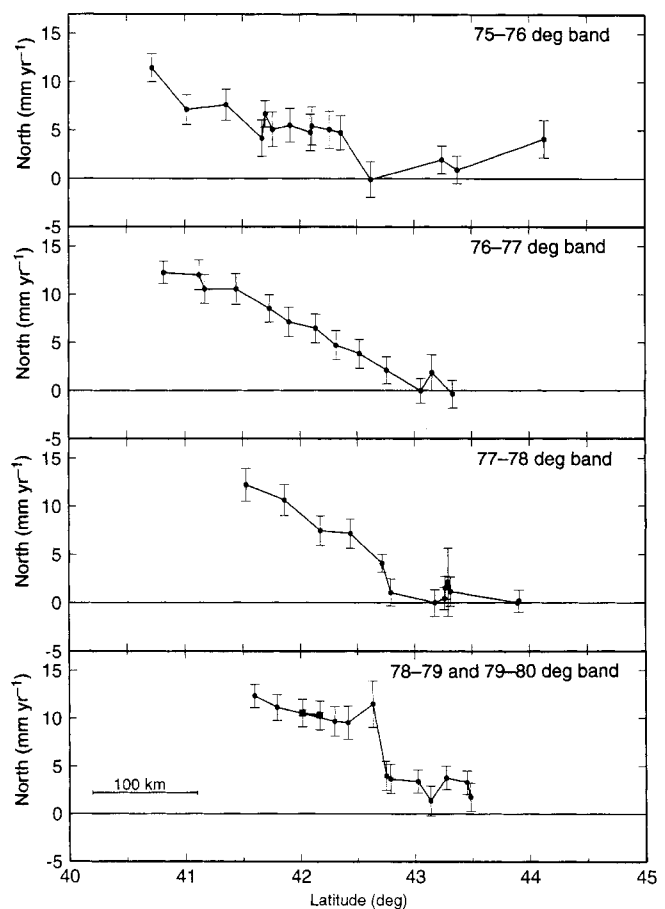


FIG. 3 Profiles of north-south components of velocity across the region along four profiles, measured with respect to station AZOK, one of the two northernmost points on the 77–78 degree band. Speeds increase southwards, showing north-south shortening across the region and, for most profiles roughly uniform strain. The step in northerly speeds on the eastern profiles may indicate localized strain (faulting at the eastern edge of the Isyk Kul basin) but may also be an error (note its large uncertainty).

mantle compensated for part of the topography<sup>35</sup>, even less shortening would have occurred, and the acceleration could be even younger. Makarov<sup>38</sup>, in fact, inferred from a synthesis of faulting that the total Cenozoic shortening across the belt does not exceed 50 km, which would call for a very recent acceleration of deformation. Regardless of the total amount of shortening, the terrain comprising the Tien Shan appears to have developed long after India collided with Eurasia, by rapid shortening distributed both within and along the margins of the belt. Timing of the change from normal to thrust faulting in Tibet, of climate change in Asia possibly due to a rapid uplift of the plateau, and of north-south shortening within the Indian plate south of India, all at  $\sim 8$  Myr, suggest that the Tibetan plateau rose abruptly by 1–2.5 km in only a few million years<sup>8,9</sup>. A roughly 10-Myr onset of rapid growth of the Tien Shan might result from the increase in the force transmitted by a higher Tibetan plateau to its surroundings<sup>9</sup>. □

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## Turtles as diapsid reptiles

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THE traditional classification of reptiles is based on a single key character, the presence and style of fenestration in the temporal region of the skull. Snakes, lizards, crocodiles, dinosaurs and others are 'diapsids', in that they have (at least primitively) two holes in the temporal region. Reptiles in which the skull is completely roofed, with no temporal fenestration, are the 'anapsids'. These include many Palaeozoic forms such as captorhinomorphs, procolophonids and pareiasaurs, but also include Testudines (turtles and tortoises). Consistent with this assumption, recent analyses of the affinities of Testudines have included Palaeozoic taxa only, placing them as akin to captorhinomorphs<sup>1</sup> or procolophonids<sup>2</sup> or nested within pareiasaurs<sup>3,4</sup>. Here we adopt a broader perspective, adding a range of Mesozoic and extant taxa to the analysis. Our result robustly supports the diapsid affinities of turtles, and so requires reassessment of the use of turtles as 'primitive' reptiles in phylogenetic reconstruction. More generally, it illustrates the difficulties of treating groups, such as the Testudines, that have extant members with peculiar morphologies that mask phylogenetic affinity; the hazards of relying on key characters such as temporal fenestra-

tion, which may mislead; the problems of outgroup choice for wide-ranging, inclusive analyses that include data from Recent and extinct groups; and the difficulties of judging the value of parsimony when applied to such inclusive analyses.

Shortly after Williston's<sup>5</sup> influential contribution to reptile classification, it became accepted that reptile interrelationships can be determined on the basis of temporal fenestration. It was recognized that two basic modes of dermatocranial reduction can be distinguished in the reptile skull: temporal fenestration, in which there are holes in the skull, and temporal emargination<sup>6</sup> in which there is an embayment of the bone covering the cheek from below and from behind. Anapsid reptiles are typically characterized by a complete roofing of the temporal region, whereas diapsid reptiles show the presence of an upper, or of an upper and a lower, temporal fenestra. Turtles became universally accepted as the only surviving clade of anapsid reptiles (subject to a variable degree of temporal emargination in derived members of the clade), despite the fact that the temporal roofing exhibited by the earliest fossil turtle, *Proganochelys*<sup>7</sup>, which has a very tall quadratojugal and an almost entirely supra-orbital position for the ventral limit of the squamosal, does not match the pattern observed in other anapsids<sup>8</sup>. The dogma of the anapsid turtle skull also neglects problems in the reconstruction of the ancestral diapsid condition. One cladistic analysis of amniote interrelationships suggests that the lower (lateral) temporal fenestra may not have evolved in the common ancestor of diapsids<sup>9</sup>. Alternatively, the description of new fossil taxa indicates the early loss of the lower (lateral) temporal fenestra in stem-group diapsids (Araucoscelidia)<sup>10</sup>, a process that also occurred convergently in crown-group diapsids (Placodontia)<sup>11</sup>. The dogma of the 'primitive' (anapsid) turtle skull was hard to reconcile with the claim that the difficulty in recognizing turtle origins is caused by the very specialized (highly derived) nature of the postcranium<sup>12</sup>.

Rejecting *a priori* the possibility of diapsid affinities of turtles, previous cladistic analyses of their phylogenetic relationships have been restricted to Palaeozoic taxa only, with *Petrolacosaurus* as the only stem-group diapsid included. Turtle relationships within anapsid reptiles have remained controversial, however. Although there has been support for a sister-group relationship of turtles with 'stem-reptiles' (Captorhinidae)<sup>1</sup>, reconsiderations of parareptile<sup>13</sup> interrelationships have found turtles to be most closely related to procolophonids<sup>2</sup> or pareiasaurs<sup>3</sup>. Turtles have recently been found to be nested within pareiasaurs<sup>4</sup>.

By contrast, the status of turtles as anapsid reptiles has long been questioned. Lakjer<sup>14</sup> concluded, after the study of the jaw adductor musculature of sauropsids, that turtles share diapsid affinities. This implied that the complete dermal roofing of the temporal region of the skull in some turtles, including *Proganochelys*<sup>7</sup>, would be secondary, as had previously been argued<sup>15</sup>. Turtles were then placed close to the lepidosaurs<sup>16</sup> (the Tuatara, lizards and snakes, and their fossil relatives): 'Those who regard the structure of the temporal region of the skull as the safest guide to affinity will naturally place the chelonians either with the primitive mammal-like reptiles or the cotylosaurs; those who hold that more reliance can be placed on the structure of the girdles and limbs will be more impressed with the affinities to the primitive diapsids such as *Sphenodon*.'

It is well known that the addition of taxa can have profound effects on cladistic analyses<sup>13,17</sup>. An analysis of cladistic interrelationships of Triassic stem-group Sauropterygia (a Mesozoic clade of secondarily marine diapsids), found turtles to share unexpected relationships. Turtles were found to be the sister-taxon of Sauropterygia<sup>18</sup>, the two clades nested within crown-group diapsids (Sauria) as the sister-group of the Lepidosauriformes<sup>19</sup>. The position of turtles as sister-group of Lepidosauriformes within crown-group diapsids (Sauria) persisted after Sauropterygia had been deleted from the analysis<sup>20</sup>.

The hypothesis of diapsid (lepidosauriform) relationships of turtles is supported by several striking developmental features of skeleton formation which turtles share with lepidosaurs<sup>20</sup>, includ-

ing the pattern of early ossification of postorbital (temporal) bones in the skull. During the early stages of ossification, the jugal shows a semilunate shape without a posterior process in both lepidosaurs<sup>21</sup> and turtles<sup>22</sup>, whereas a triradiate jugal with a posterior process may be primitive for amniotes, as both extant mammals<sup>23</sup> and crocodiles<sup>24</sup> retain this condition during early ossification of the jugal.

Other important features derive from the development of the appendicular skeleton. Turtles and squamates both lack a true radiale in the carpus, its topological equivalent being a centrale budding from the intermedium during development. Crocodiles and extant mammals share the presence of a true radiale budding from the radius<sup>25</sup>. The development of a hooked fifth metatarsal and, in particular, the pattern of its early ossification, which differs from the ossification pattern in other metatarsals, are identical in turtles and lepidosaurs<sup>20–22</sup>. And, most importantly perhaps, both turtles and lepidosaurs develop a single proximal tarsal cartilage (tarsale proximale<sup>26</sup>), formed by the coalescence of all chondrification centres of the proximal tarsus and providing the sole cartilaginous precursor of the astragalus and calcaneum ossifications, a character uniquely derived among Amniota<sup>27</sup>.

To further test the possible diapsid relationships of turtles, we compiled a data matrix for Amniota which includes a total of 33 taxa and 168 osteological characters (see Supplementary Information). Many of these characters are new, but the list also includes those characters used in earlier cladistic analysis of turtle interrelationships<sup>2,3</sup> with a few exceptions where disagreement of character interpretation prevails (such as the presumed identity of the acromion in the turtle pectoral girdle with the 'acromion' on the pareiasaur scapula<sup>3,4,28</sup>). Cladistic analysis rooting the monophyletic ingroup Reptilia<sup>2,17</sup> on Seymouridae, Diadectomorpha and Synapsida as outgroups yields two equally parsimonious trees with a tree length of 770 steps, a consistency index of 0.508, and a retention index of 0.687. The difference between the two trees involves the relative position of archosauriform taxa and does not affect the position of turtles, which remains consistent within the Diapsida. Rooting the analysis on a hypothetical all-0-ancestor (a hypothetical taxon coded 0 for all characters so that the analysis is on the presence of characters) yields four equally parsimonious trees with lack of resolution only within Archosauriformes and the relative position between Seymouridae and Diadectomorpha. Testudines is consistently the sister-group of the Sauropterygia, and these two clades are the sister-group of the Lepidosauriformes within the Sauria. The tree is shown in Fig. 1; synapomorphy listings for the labelled nodes are given in the Methods section.

The present result is superior to alternative hypotheses of turtle relationships<sup>2,3</sup> in terms of tree length, which is one measure of

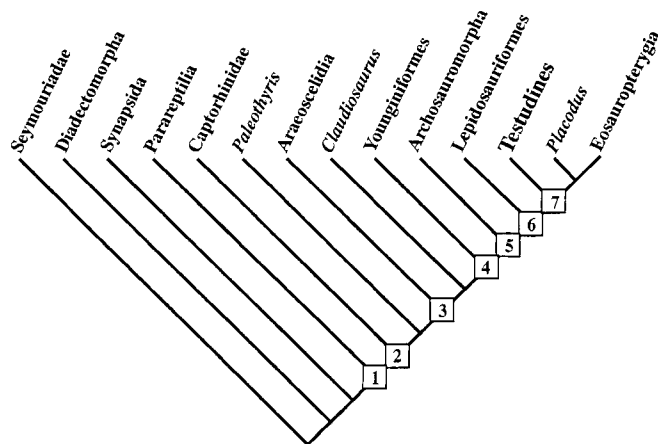


FIG. 1 Strict consensus tree of two equally parsimonious trees for amniote interrelationships. Reptilia is the ingroup, other taxa are outgroups. The analysis is based on 168 characters; tree length is 770; consistency index is 0.508; and the retention index is 0.687.

parsimony. However, the trees obtained with our data set are inferior to previous analyses in terms of consistency index, retention index and bootstrapping support, indicating a higher degree of homoplasy in our data. But this is an expected statistical result of increasing the number of characters as well as selecting numerous and diverse taxa belonging to many separate clades of amniotes for comparison.

The hypothesis that Testudines shares crown-group diapsid affinities has implications for neontologists and palaeontologists alike. Accepting turtles as the only living anapsids provided a convenient way to use the group for determination of the 'primitive' condition or character state for all kinds of comparative studies (histological, anatomical, physiological and ecological) involving reptiles. In cladistic parlance, turtles provided the ideal extant outgroup for comparative studies within Diapsida. With the possibility that Testudines are nested within crown-group diapsids, turtles no longer represent 'living fossils' among extant reptiles, and their use in comparative studies has to be thoroughly re-evaluated. Indeed, accepting Testudines as crown-group Diapsida may not only change the way we look at turtles, but may change our view of reptiles in general. □

## Methods

Cladistic analysis was based on the implementation of the software package PAUP (Phylogenetic Analysis Using Parsimony) version 3.1.1. developed by D. L. Swofford. Terminal taxa included in the analysis are the Seymouridae, Diadectomorpha, 'mammal-like reptiles' (Synapsida, that is Caseidae, Ophiacodontidae, Edaphosauridae, Sphenacodontidae, Gorgonopsia, Cynodontia), 'stem-reptiles' (Captorhinidae, *Palaeothyris*), parareptiles (Millerettidae, *Acleistorhinus*, *Lanthanosuchidae*, *Macroleter*, *Bradysaurus*, *Scutosaurus*, *Anthodon*, *Procolophonidae*, *Owenettidae*), stem-group Diapsida (*Araucoscelidia*, *Claudiosaurus*, *Younginiformes*), crown-group diapsids of the lepidosauriform clade (*Kuehneosauridae*, *Rhynchocephalia*, *Squamata*), crown-group diapsids of the archosauriform clade (*Rhynchosauria*, *Prolacertiformes*, *Trilophosaurus*, *Choristodera*, *Archisauriformes*), *Sauropterygia* (*Placodus*, *Eosauropterygia*) and turtles. For the list of the 168 osteological characters and the data matrix for the analysis see Supplementary Information. The data set was subjected to a heuristic search using the options 'stepwise addition' and 'random search' with 20 replicates. In each case the results were the same.

Synapomorphies for the numbered nodes in Fig. 1 follow DELTRAN character optimization, which favours convergence over reversals and hence provides the most conservative approach to taxon diagnosis. Homoplasy of certain characters in parareptiles is not discussed, nor are reversals within subclades of the groups diagnosed below. Unreversed synapomorphies that link turtles with successive levels of inclusiveness within the Diapsida are italicized. 1. Reptilia. Supraoccipital constricted, resulting in an open occiput; large post-temporal fenestra; suborbital fenestra present; single coronoid ossification; reduced supinator process confluent with humeral shaft; medial pedal centrale lost. 2. Eureptilia. Loss of postorbital-supratemporal contact; squamosal enters post-temporal fenestra; reduced anterior process of quadrate; supra-temporal reduced; two coracoid ossifications. 3. Diapsida. Preorbital skull length greater than postorbital skull length; frontals with posterolateral processes; upper temporal fenestra present; lower temporal fenestra present; splenial excluded from mandibular symphysis; mineralized sternum present; proximal overlap of metapodials. 4. Neodiapsida. Quadrate deeply excavated posteriorly; quadrate exposed in lateral view; stapes rod-like; dorsal process of stapes reduced; retroarticular process on lower jaw present; transverse processes present beyond the fifth caudal; fibular shaft straight; fifth metatarsal with broad base. 5. Sauria. Lacrimal duct partially bordered by maxilla; postparietals lost; supratemporal lost; opisthotic sutured to cheek; prootic contacts parietal; median wall of otic capsule ossified; sphenethmoid ossification lost; cultriform process length reduced; anterior extent of prearticular reduced; vertebral centra non-notochordal; loss of entepicondylar foramen; loss of manual perforating foramen; loss of fifth distal tarsal; hooked fifth metatarsal present. 6. Lepidosauriformes. High ascending process of maxilla present; antorbital skull length equals postorbital skull length; prefrontal in broad contact with palatine; postfrontal enters anterolateral margin of the upper temporal fenestra; loss of quadratojugal; anterior displacement of pineal foramen; lower temporal fossa open ventrally; basioccipital tubera present; loss of teeth on transverse pterygoid flange; angular narrowly exposed in lateral view; loss of dorsal intercentra; thyroid fenestra present; loss of pedal perforating foramen; loss of distal tarsal I. 7. Unnamed taxon (Testudines + Sauropterygia). Choana curves posteromedially; parasphenoid short and broad; longitudinal orientation of pterygoid flange; mineralized sternum absent; coracoid foramen bordered by scapula and coracoid; humerus not twisted; femoral distal condyles reduced; loss of both pedal centralia; limbs short and stout; manus and pes short and stout.

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## Mutations in the hepatocyte nuclear factor-1 $\alpha$ gene in maturity-onset diabetes of the young (MODY3)

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**THE disease non-insulin-dependent (type 2) diabetes mellitus (NIDDM) is characterized by abnormally high blood glucose resulting from a relative deficiency of insulin<sup>1</sup>. It affects about 2% of the world's population and treatment of diabetes and its complications are an increasing health-care burden<sup>2</sup>. Genetic factors are important in the aetiology of NIDDM, and linkage studies are starting to localize some of the genes that influence the development of this disorder<sup>3</sup>. Maturity-onset diabetes of the young (MODY), a single-gene disorder responsible for 2–5% of NIDDM, is characterized by autosomal dominant inheritance and an age of onset of 25 years or younger<sup>4–6</sup>. MODY genes have been localized to chromosomes 7, 12 and 20 (refs 5, 7, 8) and**

**clinical studies indicate that mutations in these genes are associated with abnormal patterns of glucose-stimulated insulin secretion<sup>1,9</sup>. The gene on chromosome 7 (*MODY2*) encodes the glycolytic enzyme glucokinase<sup>5</sup> which plays a key role in generating the metabolic signal for insulin secretion and in integrating hepatic glucose uptake. Here we show that subjects with the MODY3-form of NIDDM have mutations in the gene encoding hepatocyte nuclear factor-1 $\alpha$  (HNF-1 $\alpha$ , which is encoded by the gene *TCF1*). HNF-1 $\alpha$  is a transcription factor that helps in the tissue-specific regulation of the expression of several liver genes<sup>10,11</sup> and also functions as a weak transactivator of the rat insulin-I gene<sup>12</sup>.**

Linkage analysis localized *MODY3* to a 5-cM interval between the markers *D12S86* and *D12S807/D12S820* (ref. 13). A combined yeast (YAC), bacterial (BAC) and P1-derived artificial chromosome (PAC) contig spanning *D12S86* and *D12S807* (Fig. 1) was generated using information in public databases<sup>14,15</sup> and screening appropriate YAC, BAC and PAC libraries with sequence-tagged sites (STSs) from the *MODY3* region. The physical map enabled us to localize new polymorphisms as they were reported, as well as to generate new markers to pinpoint recombination events in key individuals. Such studies refined the localization of *MODY3* to the 3-cM interval between *D12S1666* and the polymorphic STS *UC-39*. Fluorescence *in situ* chromosomal hybridization using the BAC 162B15 mapped the contig to chromosome band 12q24.2 (data not shown).

This combination of genetic and physical mapping information enabled us to begin a systematic search for *MODY3*. We used a combination of approaches, including testing genes known to be on the long arm of chromosome 12 to see if they mapped into the contig, exon trapping<sup>16</sup>, and complementary DNA selection<sup>17</sup>, for which human pancreatic islet cDNA was used because insulin secretion is abnormal in *MODY3* patients<sup>9</sup>, making islets a likely site of expression of *MODY3* messenger RNA and protein. We identified 14 genes encoding known proteins (the  $\gamma$ -subunit of AMP-activated protein kinase, citron, the GTP-binding protein H-ray, paxillin, acidic ribosomal phosphoprotein P0, pancreatic phospholipase A2, splicing factor SRp30, cytochrome c oxidase subunit VIA, short-chain acyl CoA dehydrogenase, HNF-1 $\alpha$ , thyroid-receptor interactor (TRIP14) protein, Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase, P<sub>2</sub><sub>4</sub> purinoceptor and restin), five pseudogenes (with metalloproteinase-like, cell-surface heparin-binding protein-like, ribosomal protein L12-like, nucleoside diphosphate kinase-like and ADP ribosylation factor-like sequences), 12 known expressed sequence tags (ESTs) (yq81d09, yd50d03, IB383, hbc3028, yu36h05, yn75d09, yz51b06, yd88g07, ym03h09, ym30e05, WI-6178/c-01h06, WI-6239/c-04b12) and nine new ESTs (Fig. 1; K.Y., N.O., M.V., L.S. and R.D.C., manuscript in preparation).

Comparison of the sequences of pancreatic phospholipase A2,  $\gamma$ -subunit of AMP-activated protein kinase, H-ray, cytochrome c oxidase subunit VIA, acidic ribosomal phosphoprotein P0,



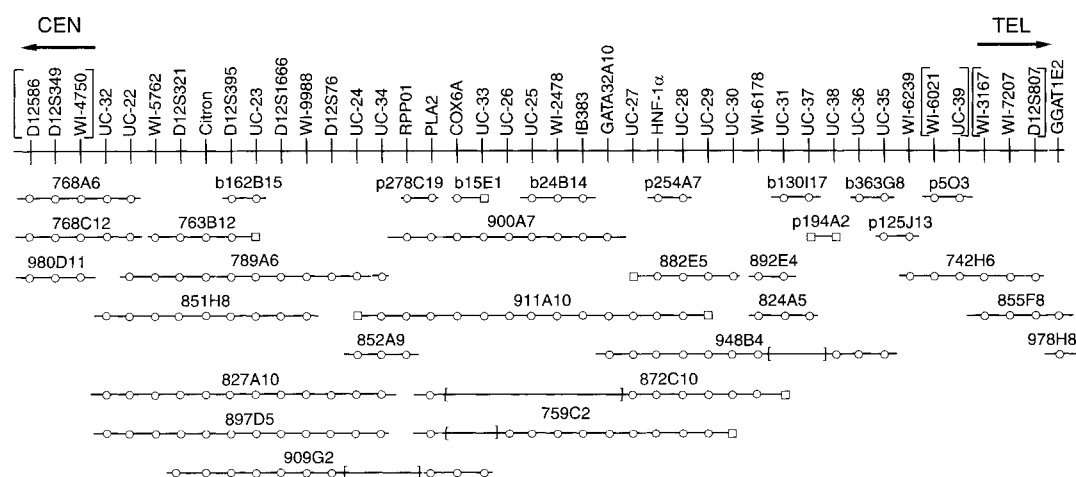


FIG. 1 Physical map of the MODY3 region of chromosome 12. YAC, BAC (b) and PAC (p) clones are represented as lines, the length of which reflects the number of included STSs and not the actual size. The physical distance between adjacent STSs has not been determined directly; STSs for which the order has not been unambiguously determined are indicated in

brackets. A circle indicates that the clone was positive for the indicated STS and a square indicates an STS derived from the end of that specific clone. Several YACs contain large internal deletions which are noted by brackets. The STSs are from the GDB and the GenBank STS databases.

paxillin, splicing factor SRp30, short-chain acyl CoA dehydrogenase and  $P_{2 \times 4}$  purinoceptor genes from MODY3 subjects and controls revealed a number of polymorphisms but no MODY3-associated mutations (data not shown).

The sequence of exon 4 of the HNF-1 $\alpha$  gene of subject EA1 (Edinburgh pedigree; Fig. 2) showed an insertion of a cytosine in codon 291 (Pro) (designated P291fsinsC), resulting in a frameshift and synthesis of a truncated mutant protein of 315 amino acids. This mutation was present in all affected members and no unaffected members of this family. It was not found either on screening 55 healthy non-diabetic white subjects (110 chromosomes). No other mutations were noted on sequencing the remaining exons. Six additional mutations were found in other MODY3 families (Table 1 and Fig. 2), all of which co-segregated with NIDDM, and did not occur in any of at least 50 healthy non-diabetic white subjects. However, there were individuals in several pedigrees (GK pedigree, III-3; Ber pedigree, V-2; and P pedigree, IV-5 and IV-6) who had inherited the mutant allele (and the at-risk chromosome-12 haplotype) but who were non-diabetic or only showed evidence of impaired glucose tolerance or diabetes during pregnancy. We would expect these individuals to develop diabetes mellitus eventually. In addition, one subject with NIDDM did not have the mutant allele (Ber pedigree, II-2). He was diagnosed with NIDDM at 65 years of age, at which time he was mildly obese with a body mass index of 27 kg m $^{-2}$ , suggesting that he had late-onset NIDDM rather than MODY. Such heterogeneity within MODY families has been noted previously<sup>5,8</sup> and is due to the high frequency of late-onset NIDDM which affects 10% or more of individuals over 65 years old<sup>18</sup>. In addition to the mutations listed in Table 1, three amino-acid polymorphisms (I/L27, A/V98 and S/N487), four silent polymorphisms (in codons for L17, G288, L459 and T515), and seven polymorphisms in introns were found in the HNF-1 $\alpha$  gene (Table 2).

HNF-1 $\alpha$  is composed of three functional domains<sup>11</sup>: an amino-terminal dimerization domain (amino acids 1–32), a DNA-binding domain with POU-like and homeodomain-like motifs (amino acids 150–280) and a carboxy-terminal

transactivation domain (amino acids 281–631). The functional form of HNF-1 $\alpha$  is a dimer, and HNF-1 $\alpha$  may form homodimers or heterodimers with the structurally related protein HNF-1 $\beta$  (ref. 19). It is not clear how mutations in the HNF-1 $\alpha$  gene can cause diabetes when present on a single allele. It is possible that a partial deficiency of HNF-1 $\alpha$  could lead to  $\beta$ -cell dysfunction and diabetes. Alternatively, mutations in HNF-1 $\alpha$  may cause diabetes by a dominant-negative mechanism<sup>20</sup> by interfering with the function of wild-type HNF-1 $\alpha$  and other proteins that act together with HNF-1 $\alpha$  to regulate transcription in the  $\beta$ -cell and/or liver. All HNF-1 $\alpha$  gene mutations so far identified would result in the synthesis of a mutant protein impaired in DNA binding or transactivation but not dimerization. These mutant proteins could form non-productive dimers with the product of the normal HNF-1 $\alpha$  allele or with other proteins such as HNF-1 $\beta$  and so impair the normal function of HNF-1 $\alpha$ .

Transgenic mice that lack HNF-1 $\alpha$  have been generated<sup>21</sup>. Homozygous HNF-1 $\alpha$ -deficient animals failed to thrive and usually died around the time of weaning. They also suffered from phenylketonuria and renal tubular dysfunction, but apparently were not diabetic as they had normal blood glucose levels and a normal response to an intravenous bolus injection of glucose, although the massive glucosuria in these animals may have reduced plasma glucose sufficiently to mask diabetes mellitus which would have been evident had renal function been normal. The insulin secretory responses of heterozygous HNF-1 $\alpha$ -deficient mice, animals that may be most similar to human subjects

TABLE 1 HNF-1 $\alpha$  gene mutations in MODY3 patients

| Family    | Location of mutation |         | Nucleotide change                                                | Amino-acid change     | Designation                 |
|-----------|----------------------|---------|------------------------------------------------------------------|-----------------------|-----------------------------|
|           | Exon                 | Codon   |                                                                  |                       |                             |
| Edinburgh | 4                    | 291     | Insertion of C                                                   | Frameshift            | P291fsinsC                  |
| A         | 7                    | 447     | CCG $\rightarrow$ CTG                                            | Pro $\rightarrow$ Leu | P447L                       |
| R         | 6                    | 379     | Deletion of CT                                                   | Frameshift            | P379fsdelCT                 |
| GK        | Intron 9             |         | GT $\rightarrow$ AT<br>at splice donor site                      |                       | IVS9nt + 1G $\rightarrow$ A |
| Ber       | 9                    | 547/548 | Deletion of TG<br>AG $\rightarrow$ GG<br>at splice acceptor site | Frameshift            | T547E548fsdelTG             |
| P         | Intron 5             |         |                                                                  |                       | IVS5nt – 2A $\rightarrow$ G |
| H         | 2                    | 131     | CGG $\rightarrow$ CAG                                            | Arg $\rightarrow$ Gln | R131Q                       |

The missense mutations P447L and R131Q are of residues that are conserved in human, rat, mouse, hamster, chicken and salmon HNF-1 $\alpha$  and in human HNF-1 $\beta$ .

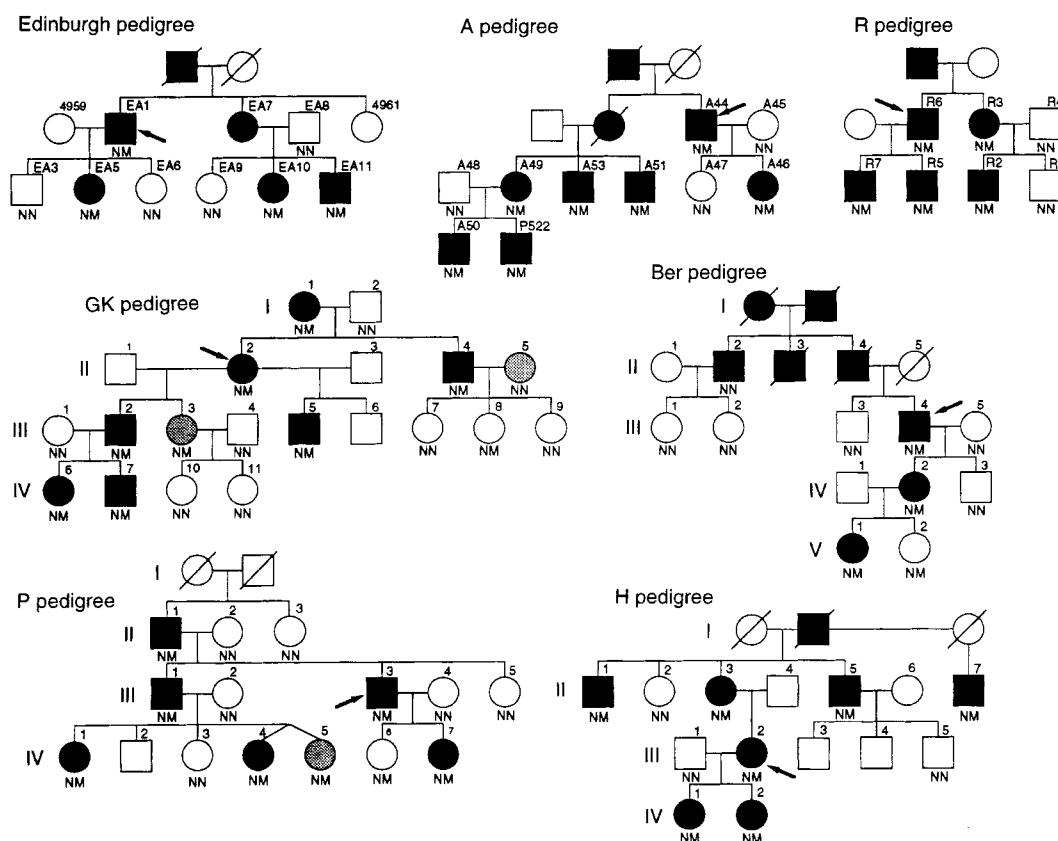


FIG. 2 MODY families with mutations in the HNF-1 $\alpha$  gene. Individuals with MODY/NIDDM are indicated by black symbols, and those with gestation-onset diabetes or impaired glucose tolerance by shaded symbols. Non-diabetic individuals are indicated by white symbols. Arrows indicate the individual from each pedigree who was screened for mutations. The HNF-1 $\alpha$  genotype of each individual is indicated below the symbol: N, normal; M, mutant.

with HNF-1 $\alpha$  mutations and MODY, were not reported. In view of the present findings that mutations in the HNF-1 $\alpha$  gene can cause early-onset NIDDM, more detailed evaluation of pancreatic  $\beta$ -cell and liver function in HNF-1 $\alpha$ -deficient mice is indicated.

Although mutations in the HNF-1 $\alpha$  gene are associated with MODY, there is no evidence from linkage studies that common mutations in this gene are a significant factor contributing to the development of the late-onset form(s) of NIDDM in Mexican Americans, non-Hispanic whites or Japanese (refs 3, 22, and our unpublished results). One study has suggested that markers near *MODY3* may be linked to NIDDM in a small subset of families having a low insulin-secretory response and from an isolate population<sup>23</sup>. The identification of *MODY3* will allow these families to be tested for mutations in the HNF-1 $\alpha$  gene. Although there is no evidence indicating that mutations in the HNF-1 $\alpha$  gene are associated with susceptibility to the common late-onset form(s) of NIDDM, acquired defects in HNF-1 $\alpha$  could contribute to the dysfunction of  $\beta$ -cells that characterizes this disorder as the  $\beta$ -cell defects in subjects with MODY and NIDDM are similar<sup>9,24</sup>. The demonstration that mutations in HNF-1 $\alpha$  and the functionally related transcription factor HNF-4 $\alpha$  (refs 25, 26) are associated with MODY should focus attention on the role of these proteins in determining normal pancreatic  $\beta$ -cell function.  $\square$

## Methods

**Isolation and partial sequence of the human HNF-1 $\alpha$  gene.** The PAC clone 254A7, containing the human HNF-1 $\alpha$  gene, was isolated from a library (Genome Systems) by screening PAC DNA pools using polymerase chain reaction (PCR) and the primers HNF1P1 (5'-TACACCACTCTGGCAGCCACACT-3') and HNF1P2 (5'-CGGTGGGTACATTGGTGACAGAAC-3'). The sequences of the exons and flanking introns were determined after subcloning fragments of 254A7 into pGEM-4Z (Promega Biotech) or pBluescript SK+ (Stratagene), then sequencing using primers based on the sequence of human HNF-1 $\alpha$  cDNA<sup>27,28</sup>, and selected using the conserved exon-intron organization of the mouse and rat genes<sup>29</sup> as a guide. The human gene consists of 10 exons, with introns 1–8 located in the same positions as in the rat and mouse genes<sup>29</sup>. Intron 9 interrupts codon 590 (phase 1) and is not present in the rat and mouse genes but does occur in the chicken gene<sup>30</sup>. The sequences of the exons and

TABLE 2 DNA polymorphisms in human HNF-1 $\alpha$  gene

| Location |         |                                 |                |
|----------|---------|---------------------------------|----------------|
| Exon     | Codon   | Nucleotide change               | Frequency      |
| 1        | 17      | CTC(Leu) $\rightarrow$ CTG(Leu) | C 0.57, G 0.43 |
| 1        | 27      | ATC(Ile) $\rightarrow$ CTC(Leu) | A 0.63, C 0.37 |
| 1        | 98      | GCC(Ala) $\rightarrow$ GTC(Val) | C 0.98, T 0.02 |
| 4        | 288     | GGG(Gly) $\rightarrow$ GGC(Gly) | G 0.67, C 0.33 |
| 7        | 459     | CTG(Leu) $\rightarrow$ TTG(Leu) | C 0.63, T 0.37 |
| 7        | 487     | AGC(Ser) $\rightarrow$ AAC(Asn) | G 0.72, A 0.28 |
| 8        | 515     | ACG(Thr) $\rightarrow$ ACA(Th)  | G 0.79, A 0.21 |
| Intron 1 | nt - 91 | A $\rightarrow$ G               | A 0.88, G 0.12 |
| Intron 1 | nt - 42 | G $\rightarrow$ A               | G 0.66, A 0.34 |
| Intron 2 | nt - 51 | T $\rightarrow$ A               | T 0.85, A 0.15 |
| Intron 2 | nt - 23 | C $\rightarrow$ T               | C 0.88, T 0.12 |
| Intron 5 | nt - 47 | C $\rightarrow$ T               | C 0.99, T 0.01 |
| Intron 7 | nt 7    | G $\rightarrow$ A               | G 0.57, A 0.43 |
| Intron 9 | nt 44   | C $\rightarrow$ T               | C 0.96, T 0.04 |
| Intron 9 | nt - 24 | T $\rightarrow$ C               | T 0.59, C 0.41 |

The frequency of each polymorphism was determined by genotyping 20–56 unrelated normal healthy white subjects. DNA polymorphisms found in introns are noted with respect to the splice donor or acceptor site. nt, Nucleotide.

adjacent introns have been deposited in the GenBank database under accession numbers (U72612–8).

**Screening of HNF-1 $\alpha$  gene for mutations.** The ten exons and flanking introns of the HNF-1 $\alpha$  gene were amplified using PCR and specific primers (see Supplementary Information). PCR conditions were denaturation at 94 °C for 5 min following by 35 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 45 s, and final extension at 72 °C for 10 min. PCR products were purified using a Centricon-100 membrane (Amicon) and sequenced directly from both ends using specific primers (see Supplementary Information) an AmpliTaq FS Dye Terminator cycle sequencing kit and ABI Prism 377 DNA sequencer. The sequence of each mutation was confirmed by cloning the PCR product into the *HincII* site of pGEM-3Z (Promega) and sequencing clones representing both alleles. The presence of the specific mutation in other

family members and unrelated non-diabetic white controls was assessed by amplifying and directly sequencing the appropriate exon.

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## Mutations in the hepatocyte nuclear factor-4 $\alpha$ gene in maturity-onset diabetes of the young (MODY1)

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THE disease maturity-onset diabetes of the young (MODY) is a genetically heterogeneous monogenic form of non-insulin-dependent (type 2) diabetes mellitus (NIDDM), characterized by early onset, usually before 25 years of age and often in adolescence or childhood, and by autosomal dominant inheritance<sup>1</sup>. It has been estimated that 2–5% of patients with NIDDM may have this form of diabetes mellitus<sup>2,3</sup>. Clinical studies have shown that prediabetic MODY subjects have normal insulin sensitivity but suffer from a defect in glucose-stimulated insulin secretion, suggesting that pancreatic  $\beta$ -cell dysfunction rather than insulin resistance is the primary defect in this disorder<sup>4,5</sup>. Linkage studies have localized the genes that are mutated in MODY on human chromosomes 20 (MODY1)<sup>6</sup>, 7 (MODY2)<sup>2</sup> and 12 (MODY3)<sup>7</sup>, with MODY2 and MODY3 being allelic with the genes encoding glucokinase<sup>2</sup>, a key regulator of insulin secretion, and hepatocyte nuclear factor-1 $\alpha$  (HNF-1 $\alpha$ )<sup>8</sup>, a transcription factor involved in tissue-specific regulation of liver genes but also expressed in pancreatic islets, insulinoma cells and other tissues. Here we show that MODY1 is the gene encoding HNF-4 $\alpha$  (gene symbol, TCF14), a member of the steroid/thyroid hormone receptor superfamily and an upstream regulator of HNF-1 $\alpha$  expression<sup>9–11</sup>.

The R-W pedigree (Fig. 1), which includes more than 360 members spanning 6 generations and 74 members with diabetes, including those with MODY, has been studied prospectively since 1958 (ref. 1). The members of this family are descendants of a couple that emigrated from East Prussia to Detroit, Michigan in 1861 with their four sons, three of whom were diabetic, and five

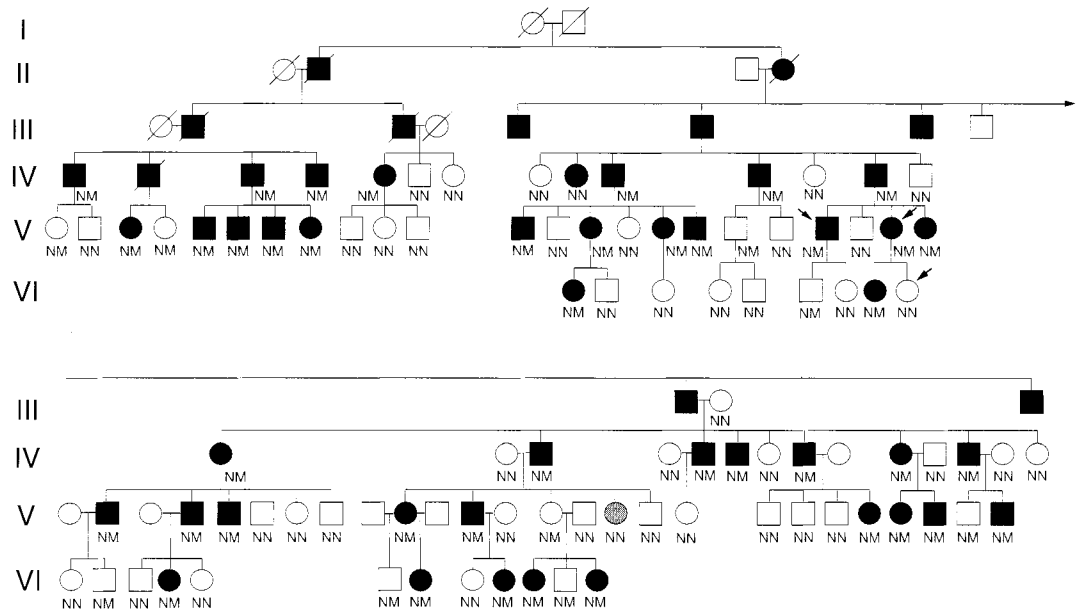
daughters, one of whom was diabetic (refs 1 and 12, and S.S.F., unpublished results). Linkage studies have shown that the gene responsible for MODY in this family, MODY1, is tightly linked to markers in the chromosome 20 band 20q12–q13.1, with a multi-point lod score >14 in those branches of the family in which MODY is segregating<sup>6,13</sup>. The analysis of key recombinants in the R-W pedigree localized MODY1 to a 13-cM interval (~7 megabases (Mb)) between markers D20S169 and D20S176, an interval which also includes the gene encoding HNF-4 $\alpha$  (ref. 14). The demonstration that mutations in the HNF-1 $\alpha$  gene are the cause of the MODY3 form of NIDDM<sup>8</sup> prompted us to screen the HNF-4 $\alpha$  gene for mutations in the R-W pedigree.

The 11 exons of the HNF-4 $\alpha$  gene of two affected (V-20 and 22) and one unaffected (VI-9) subject from the R-W pedigree (Fig. 1) were amplified and the products of polymerase chain reaction (PCR) sequenced directly. The sequences were identical to one another and the complementary DNA<sup>15</sup>, except for C  $\rightarrow$  T substitutions in codons 130 (exon 4) and 268 (exon 7). The C  $\rightarrow$  T substitution in codon 130 resulted in a Thr(ACG)  $\rightarrow$  Ile(ATT) substitution and is a polymorphism (T/I130) with a frequency of the Ile allele in a group of 55 unrelated nondiabetic non-Hispanic white subjects of 5%. The C  $\rightarrow$  T substitution in codon 268 generated a nonsense mutation CAG(Gln)  $\rightarrow$  TAG(AM) (Q268X). In the R-W pedigree, Ile 130 and the amber mutation at codon 268 were present on the same allele.

The Q268X mutation created a digestion site for the restriction enzyme *Bfa*I; digestion of the normal allele generated fragments of 281 and 34 base pairs (bp), whereas the mutant allele generated fragments of 152, 129 and 34 bp. The Q268X mutation cosegregated with the at-risk haplotype and NIDDM in the R-W pedigree (Fig. 1) and was not observed on screening 108 healthy non-diabetic non-Hispanic white subjects (216 normal chromosomes). Seven subjects in the R-W pedigree who inherited the mutant allele (V-18, 37 and 48; VI-6, 11, 15 and 20) have normal glucose tolerance (Fig. 1). Five of these subjects (V-48, and VI-6, 11, 15 and 20) are less than 25 years old and thus are still within the age range when diabetes usually develops in at-risk individuals in this family. Of the others, subject V-18 is 44 years old and has had normal glucose levels in all oral glucose tolerance tests; and subject V-37, who is 36 years old, showed impaired glucose tolerance in one test and one diabetic response at 16–17 years old, but for the past 19 years each glucose tolerance test has been normal, even though she has a low insulin response to orally administered glucose. V-37 is lean and active, and showed increased sensitivity to insulin during a frequently sampled intravenous glucose tolerance test; during a prolonged low-dose glucose infusion, she became markedly hyperglycaemic<sup>4,5</sup>. Two subjects (V-1 and V-4) who have the mutation were considered to be non-diabetic on the basis of their medical history, but their



FIG. 1 The R-W pedigree, modified and abbreviated from ref. 14. Individuals with MODY/NIDDM are indicated by black symbols and non-diabetic individuals by white symbols. Subject V-39 has insulin-dependent diabetes mellitus. Arrows indicate the three subjects (two affected and one unaffected) who were screened for mutations in the HNF-4 $\alpha$  gene. The HNF-4 $\alpha$  genotype of each individual, if known, is indicated below the symbol: N, normal; M, Q268X mutation.



status needs to be evaluated by oral glucose tolerance testing. Our results indicate that the nonsense mutation in the HNF-4 $\alpha$  gene in the R-W pedigree is highly but not completely penetrant, although the age of diabetes onset is variable.

In addition to subjects who inherited the Q268X mutation but are at present non-diabetic, there are subjects in the R-W pedigree who have NIDDM but did not inherit the Q268X mutation or at-risk haplotype. Subject IV-9 was diagnosed with NIDDM at 48 years of age and was hyperinsulinemic, a diagnosis consistent with late-onset NIDDM rather than MODY. We also tested her six children, one of whom had NIDDM and another impaired glucose tolerance, and all had two normal alleles (data not shown). Likewise, we tested 10 children of subject III-7, five of whom had NIDDM, and none had inherited the Q268X mutation, suggesting that the NIDDM in this branch of the R-W family is of a different aetiology. The five non-diabetic children of III-11 were also tested for the Q268X mutation and all were normal. The presence of both MODY and late-onset NIDDM in the R-W family has been noted previously<sup>6</sup>. The MODY phenotype results from a mutation in the HNF-4 $\alpha$  gene. The cause(s) of the late-onset NIDDM is unknown. Finally, subject V-39, who has insulin-dependent diabetes mellitus, did not inherit the Q268X mutation, suggesting that mutations in the HNF-4 $\alpha$  gene do not increase the risk of developing this form of diabetes mellitus.

HNF-4 $\alpha$  is a member of the steroid/thyroid hormone receptor superfamily and is most highly expressed in liver, kidney and intestine<sup>9,16</sup>. It is also expressed in pancreatic islets and insulinoma cells (ref. 17, and K.Y., unpublished results). HNF-4 $\alpha$  is a key regulator of hepatic gene expression and is a major activator of HNF-1 $\alpha$ , which in turn activates the expression of a large number of liver-specific genes, including those involved in glucose, cholesterol and fatty acid metabolism<sup>9,10</sup>. Its expression in kidney, intestine and pancreatic islets implies that it is important in tissue-specific regulation of gene expression in these tissues as well, although its specific function in non-hepatic tissues has not been addressed. Homozygous loss of functional HNF-4 $\alpha$  protein causes embryonic lethality characterized by defects in gastrulation, underscoring the key role played by this transcription factor in development and differentiation<sup>18</sup>. The phenotype of the heterozygous animals was not described and further studies are necessary to determine whether they represent a mouse model of MODY.

The importance of HNF-4 $\alpha$  in regulating hepatic gene expression is well established<sup>9,10</sup>, but the role it plays with HNF-1 $\alpha$ , the MODY3 product and a downstream target of HNF-4 $\alpha$ , in regulat-

ing gene expression in the insulin-secreting pancreatic  $\beta$ -cell is still unclear, although HNF-1 $\alpha$  is known to be a weak transactivator of the insulin gene<sup>19</sup>. The mechanism by which mutations in HNF-4 $\alpha$  result in an autosomal dominant form of NIDDM characterized by pancreatic  $\beta$ -cell dysfunction is not known. The nonsense mutation in HNF-4 $\alpha$  found in the R-W family is predicted to result in the synthesis of a protein of 267 amino acids with an intact DNA-binding domain. However, it is missing the regions involved in dimerization and transcriptional activation in other members of the steroid/thyroid hormone superfamily<sup>20-23</sup> and so is probably unable to dimerize or bind to its recognition site and activate transcription. We propose that the dominant inheritance is due to a reduction in the amount of HNF-4 $\alpha$  *per se*, rather than to a dominant-negative mechanism. A decrease in functional HNF-4 $\alpha$  appears to have a critical effect on  $\beta$ -cell function, perhaps because of decreased HNF-1 $\alpha$  expression; mutations in this gene also lead to MODY<sup>8</sup>. Prediabetic subjects with mutations in either the HNF-4 $\alpha$  or HNF-1 $\alpha$  genes exhibit similar abnormalities in glucose-stimulated insulin secretion, with normal insulin secretion rates at lower glucose concentrations but lower-than-normal rates as the glucose concentration increases<sup>5,24</sup>, a result consistent with HNF-4 $\alpha$  and HNF-1 $\alpha$  affecting a common pathway in the pancreatic  $\beta$ -cell. The absence of overt hepatic, renal or gastrointestinal dysfunction in affected members of the R-W pedigree suggests that the levels of HNF-4 $\alpha$  in these tissues, although possibly lower than normal, are sufficient to ensure normal function, or that alternative pathways are sufficient for expression of key genes. However, detailed investigations of hepatic glucose production and metabolism have not been made in subjects from the R-W pedigree and it is possible that subtle alterations in these processes may be operating.

The demonstration that MODY can result from mutations in the HNF-1 $\alpha$  and HNF-4 $\alpha$  genes suggests that this form of NIDDM is primarily a disorder of abnormal gene expression. Genes encoding other proteins in the HNF-1 $\alpha$ /HNF-4 $\alpha$  regulatory cascade<sup>10</sup> should be considered as candidates for other forms of MODY and/or late-onset NIDDM. The role of HNF-4 $\alpha$  in the development of the more common late-onset NIDDM is unknown. There is no evidence for linkage of markers flanking the HNF-4 $\alpha$  gene with late-onset NIDDM in Mexican Americans<sup>25</sup> or Japanese (ref. 26, and N. Iwasaki and G.I.B., unpublished results), implying that mutations in the HNF-4 $\alpha$  gene are unlikely to be a significant genetic factor contributing to the development of late-onset NIDDM. However, acquired defects in HNF-4 $\alpha$  expression may contribute, at least in part, to the  $\beta$ -cell

dysfunction that characterizes late-onset NIDDM<sup>27</sup>, especially if it is central in regulating gene expression in the pancreatic  $\beta$ -cell, as suggested by the association of mutations in the HNF-4 $\alpha$  gene with MODY. Furthermore, the similarity between HNF-4 $\alpha$  and ligand-dependent transcription factors raises the possibility that HNF-4 $\alpha$  and the genes it regulates respond to an unidentified ligand. The identification of this ligand could lead to new treatments for diabetes. □

## Methods

**Isolation and partial sequence of the human HNF-4 $\alpha$  gene.** Three PAC clones (114E13, 130B8 and 207N8) containing the human HNF-4 $\alpha$  gene were isolated from a library (Genome Systems) by screening DNA pools by PCR with the primers HNF4-1 (5'-CACCTGGTGATCAGTGGTC-3') and HNF4-2 (5'-GTAAGGCTCAAGTCATCTCC-3'). The partial sequence of the gene was determined after amplifying PAC 114E13 and genomic DNA with specific primers whose sequences were based on the human cDNA<sup>15,28</sup> and selected using the exon-intron organization of the mouse HNF-4 $\alpha$  gene<sup>29</sup> as a guide. PCR products were sequenced using an AmpliTaq FS Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 377 DNA Sequencer. This gene consists of 11 exons, with the introns being located in the same positions as in the mouse gene<sup>29</sup>. Alternative splicing generates a family of mRNAs, HNF-4 $\alpha$ 1, 2 and 4, the

latter two of which contain inserts of 30 and 90 nucleotides, respectively<sup>15,28,29</sup>. The sequence of exon 1B, the exon encoding the insertion in HNF-4 $\alpha$  mRNA, revealed an additional T between nucleotides 219 and 220 in both alleles of five unrelated individuals (10 chromosomes) not present in the cDNA sequence<sup>28</sup>, which causes a frameshift and generates a protein of 98 amino acids whose function is unknown. The sequences of the exons and adjacent introns have been deposited in the GenBank database (accession numbers, U72959–U72969).

**Screening of the HNF-4 $\alpha$  gene for mutations.** The eleven exons and flanking introns were amplified using PCR and specific primers (the primer sequences are described in Supplementary Information). PCR conditions were denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. PCR products were purified using a Centricon-100 membrane (Amicon) and sequenced directly from both ends using an AmpliTaq Cycle Sequencing Kit and ABI Prism 377 DNA sequencer. The sequence of the Q268X mutation was confirmed by cloning the exon-7 PCR product into pGEM-T (Promega) and sequencing individual clones. The presence of the C → T mutation in codon 268 in members of the R-W pedigree and a group of unrelated non-diabetics was assessed by amplifying exon 7, digesting with *Bfal*, and separation of the digested PCR product on a 3% agarose gel. The frequency of the T/130 polymorphism was determined by amplification of exon 4 and digestion with *BstBI*, which does not cleave the Thr allele but cleaves the Ile allele into fragments of 184 and 87 bp.

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## Role of learning in three-dimensional form perception

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ONE of the most remarkable characteristics of the human visual system is its ability to perceive specific three-dimensional forms in single two-dimensional contour images. This has often been attributed to a few general purpose and possibly innately specified shape biases<sup>1–6</sup>, such as those favouring symmetry and other structural regularities (Fig. 1). An alternative approach proposed by the early empiricists<sup>7–10</sup> and since tested<sup>11</sup> suggests that this ability may also be acquired from visual experience, with the three-dimensional percept being the manifestation of a learned association between specific two-dimensional projections and the correlated three-dimensional structures. These studies of shape learning have been considered inconclusive, however, because their results can potentially be accounted for as cognitive decisions that might have little to do with shape perception *per se*. Here we present an experimental system that enables objective verification of the role of learning in shape perception by render-

ing the learning to be perceptually manifest. We show that the human visual system can learn associations between arbitrarily paired two-dimensional pictures and (projectionally consistent) three-dimensional structures. These results implicate high-level recognition processes in the task of shape perception.

Our experiment comprised a training and a test phase (Fig. 2). For use as stimuli, we generated random two-dimensional (2-D) line-drawings and assigned them arbitrary three-dimensional (3-D) structures. During the training phase, subjects were shown one such object rocking through an angle of  $\pm 20$  degrees about a frontoparallel horizontal axis passing through its centroid (we shall refer to this as the 'training' object). This was to allow the subjects to observe the correlation between the object's mean-angle projection and its associated 3-D structure through the kinetic-depth effect (KDE)<sup>12,13</sup>. The test phase commenced five seconds after the training session and was intended to assess subjects' shape learning. Subjects were shown either the training object rocking back and forth, or another object that had the same mean-angle projection but a completely different 3-D structure (the 'test' object). They were asked to indicate whether the objects looked rigid or non-rigid. We expected that if the subjects had indeed learned an association between the training object's mean-angle projection and its 3-D structure, then, when presented with a test object having the same mean-angle projection, they would perceptually impose on it the learned 3-D structure and the observed motion pattern would be mapped onto this learned structure. If in fact the test object had a different 3-D structure, the sequence would appear to depict a non-rigid deformation. We

also expected that switching the roles of the training and test objects across different populations of subjects would lead to reversals in the pattern of results. Furthermore, subjects who had not undergone the training session were not expected to perceive non-rigidity, because of the well known bias towards rigidity<sup>12,13</sup>.

We divided our twelve subjects into three mutually exclusive equally sized groups. Members of the first group individually underwent a training phase and then a test session. Each subject was tested with ten different objects, each object being used only once. Members of the second population served as controls and did not undergo training sessions for any of the ten objects used. The procedure for the third population was identical to that for the first, apart from having the roles of the training and test objects reversed.

Figure 3 shows the results from the three populations. The results are plotted as the percentage of trials (over all objects) during which subjects perceived non-rigidity in the object depicted in the motion sequence. Members of population (1) show a clear effect of visual experience (Fig. 3a). Whereas the training object appeared to be non-rigid for an average (across all four subjects) of 7.5% of the presentations, the test object (rotating about the same axis) appeared to be non-rigid in ~40% of the presentations. Members of population (2), on the other hand, perceived both the training and the test objects as being rigid in most presentations (Fig. 3b). Members belonging to population (3) gave a reverse pattern of results (Fig. 3c).

The marked differences in the results of populations (1), (2) and (3) attest to the strong influence of visual experience on subsequent perception of 3-D form. We conclude that it is possible for the human visual system to learn associations between arbitrary 2-D projections and 3-D structures, and that this learning subsequently influences 3-D form perception. At least for the kinds of objects we consider here, mutual temporal correlation seems to be more important for the formation of such associations than any intrinsic structural characteristics of the 2-D and 3-D shapes. Supporting this interpretation, subjects reported recognizing the test object as being the one they had seen during the training session.

To characterize subjects' shape-learning better, we tested their performance under a few additional test conditions. As Fig. 3d

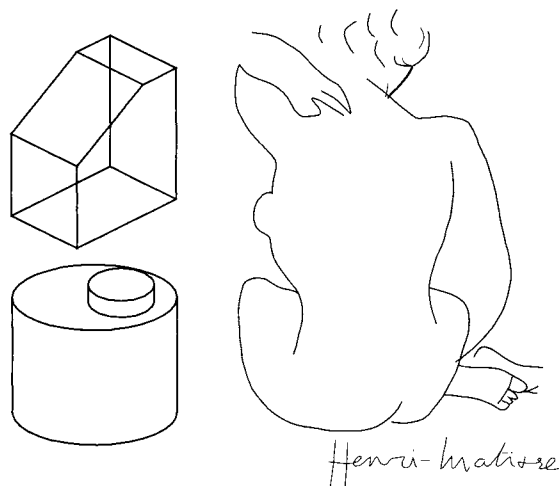


FIG. 1 These 2-D images convey a vivid sense of three-dimensionality. It is unclear whether the human visual system overcomes the underconstraint inherent in achieving this percept by the use of a few fixed and innately specified shape biases<sup>1-6</sup>, or by learning associations between specific 2-D projections and the correlated 3-D structures<sup>7-10,4-6</sup>. Computational schemes that employ a small set of prespecified shape biases have been able to mimic human 3-D shape perception with images of relatively regular geometric objects, such as the two shown on the left. They prove ineffective, however, in more general domains that might contain images such as the one shown on the right.

shows, the learning is long-lasting. Two subjects from population (1) who were trained with 10-min sequences for each of five objects (five training sessions evenly spaced over 4 hours; every session comprised 2-min sequences for each of the five objects) showed significant effects of learning upon being tested a day later. The learning also seems to be object-specific in that the percept of non-rigidity declines with the addition of 2-D positional noise to the vertices of the original projection (Fig. 3e). However, transformations that preserve the shape of the training projection, such as image scaling, do not have a large effect on subjects' responses (Fig. 3f).

In the light of these results, an important issue that needs to be addressed is how the learned 3-D structure is represented by the visual system. The two main possibilities are: either the 3-D structure can be represented 'explicitly' as an object-centred CAD-like model<sup>14</sup>, or it may be stored 'implicitly'—for instance, as a viewer-centred depth structure coupled with knowledge of how its projection transforms upon rotation about the training axis. To distinguish between these two hypotheses, we repeated our experiments with new test sequences that showed the test object rocking about an axis different from the training axis. For an explicit 3-D representation, we expected that the learning would manifest itself in the new test sequences and the percept of non-rigidity would be undiminished. An implicit 3-D representation, however, would predict a decrement in the percept because of a lack of transfer of learning across different rotation axes. As Fig. 3g shows, the percept of non-rigidity obtained with the new test sequences was significantly diminished relative to the original test sequence. This lends support to the idea of implicit 3-D representations, which is consistent with the suggestions of view-based representations for recognizing 3-D structures<sup>15-18</sup> and demonstrations of the ability of neurons in the infero-temporal cortex to code together temporally correlated frames in a motion sequence<sup>19,20</sup>.

Figure 4 shows two illusions related to the ideas we have presented here. Figure 4a and b shows a situation in which a completely rigid wireframe object resembles a person, which when rocked around the vertical axis is perceived as a person walking (a

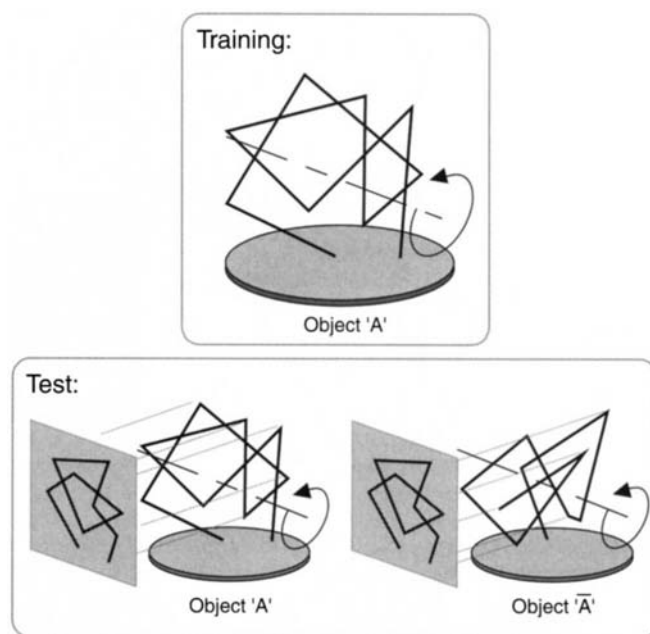


FIG. 2 Our experimental design. During the training phase, subjects saw a motion sequence of a rocking rigid 3-D object. In the subsequent test phase, subjects either saw motion sequences of the training object or of novel rigid objects, some of which had the same mean-angle projection as the training object. They were asked to report whether the objects looked rigid or non-rigid.



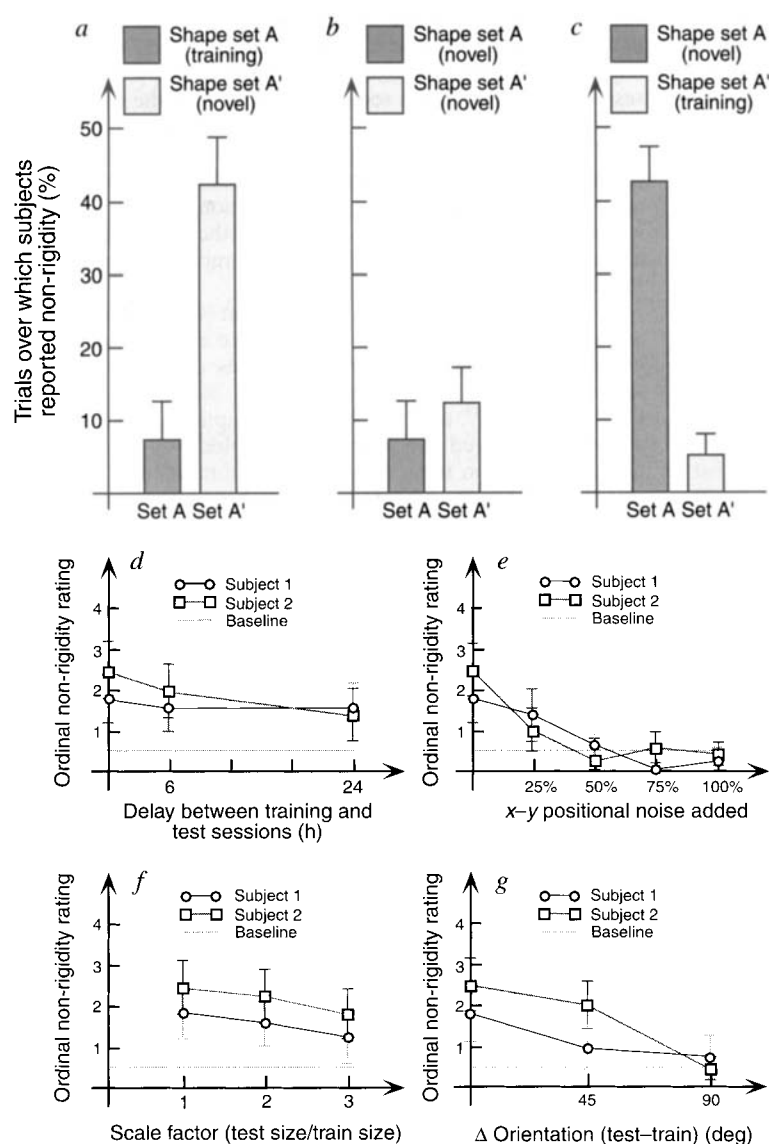


FIG. 3 Results from our three populations of subjects. Subjects were asked to indicate whether the objects shown in the motion sequences looked rigid or non-rigid. *a*, Results from population (1) on training and test objects. *b*, Results from population (2). Subjects in this group had not undergone any training sessions. *c*, Results from population (3). The training and test objects were switched for this population relative to population (1). For the experiments whose results are shown in *d*–*g*, subjects were instructed to rate the perceived non-rigidity on an ordinal scale with a score of zero corresponding to a very rigid percept, and '4' to a very non-rigid one. The grey horizontal line in all graphs is the baseline rating from a control group that did not undergo any training. The data are reported for one naive subject and one author. *d*, Persistence of shape-learning over time. *e*, The effect of adding 2-D positional noise to the mean projection of the test object. *f*, Effect of changes in image scale on the percept of non-rigidity. The training objects used here subtended, on an average, a visual angle of 2.5 deg. *g*, Decrement of the non-rigidity percept with changes in the rotation axis (angles indicate orientation differences in the image plane).

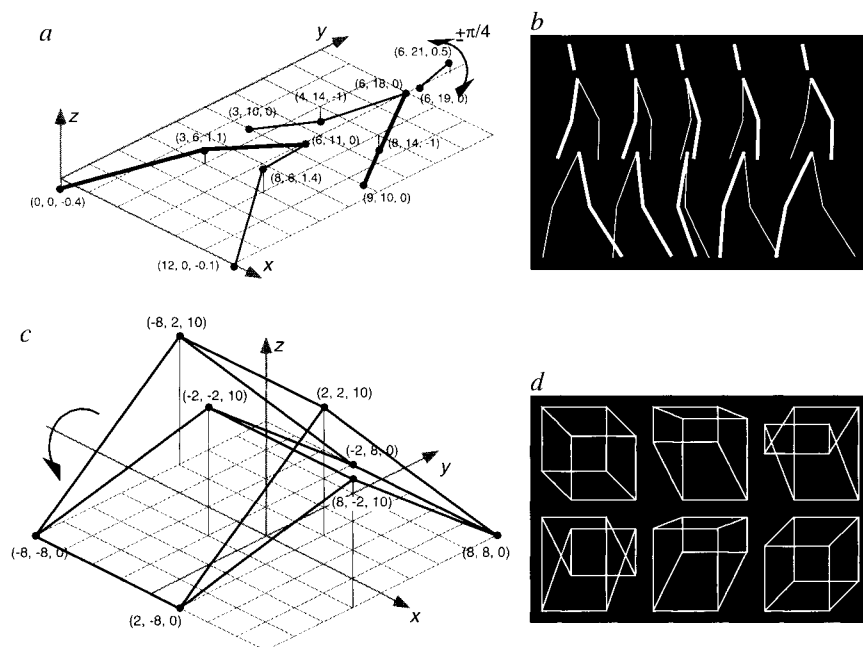


FIG. 4 Two illusions related to the ideas presented here. *a*, This rigid structure, when rocked back and forth through an angle of 90 deg about the vertical axis, is perceived as a human walking (a non-rigid interpretation). *b*, Frames from the motion sequence. This mismatch between reality and perception is probably due to the recognition of the figure as a human, which leads the visual system to interpret the motion patterns in terms of how the human structure is expected to change. This expectation is likely to have been learned through visual experience. The same vertex set, when not recognized as a human (say, when the vertices are connected up randomly) appears rigid upon rotation. *c*, An object that projects to a cube (from a specific viewpoint) but has a very different 3-D structure. *d*, A motion sequence of this object, that includes the cube-like projection, appears to depict a highly non-rigid object. With the vertices connected randomly, the percept of rigidity is restored. Just as for *a*, we suggest that the perceived non-rigidity here is likely to be due to the mismatch between the observer's expectations and the presented sequence. It is not clear whether the shape expectation for a cube (and other simple geometric objects) is learned during an observer's lifetime, or is innate. (Copies of these sequences are available from the authors.)

non-rigid interpretation). In this case, the association learned in the course of our daily experience overrides the general bias towards a rigid interpretation. Figure 4c shows a non-cuboidal object whose mean 2-D projection is the same as a cube's. A KDE sequence of this object rotating (Fig. 4d) looks highly non-rigid. However, if we connect the vertices of this object in a random fashion so that it no longer looks like a cube, thus weakening the shape expectations, its structural rigidity becomes evident.

The idea that 3-D shape perception might be mediated, at least in part, by object-specific learning has a direct corollary: 3-D shape perception may sometimes follow recognition rather than precede it (as the visual system has to be able to recognize the 2-D projection to access its associated 3-D structure). This idea (also suggested by Cavanagh<sup>21</sup>) runs contrary to the traditionally assumed hierarchy of visual processing, according to which recognition depends upon the results of the 3-D-shape recovery process<sup>22</sup>. It is natural to ask whether the learning reported here could generalize to novel objects of the same object class, as suggested by a computational scheme based on linear combinations of prototypes (ref. 23 and included references). Preliminary experiments to be reported elsewhere suggest that this is indeed the case and that the object-specific learning we describe can generalize to similar objects. □

## Methods

Subjects were trained on a 60-s sequence showing a randomly constructed rigid 3-D object rocking back and forth through an angle of 40 degrees with an angular speed of 30 deg per second. The test objects rocked back and forth through an angle of 20 deg, with an angular speed of 30 deg per second for 6 s. All sequences were presented on a Silicon Graphics workstation. The stimuli had the form of a thin wire randomly bent at seven arbitrary places along its length, displayed using anti-aliased white segments on a black background. There were no occlusion or shading cues. On average, they subtended a visual angle of 6 deg from a viewing distance of 80 cm. The objects were selected to yield a rigid percept to a group of three naive observers and also to ensure that they did not present views with accidental alignments of the constituent segments. All subjects except one (author P.S.) were naive as to the purpose of the experiment.

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# Estimation of self-motion by optic flow processing in single visual interneurons

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**HUMANS, animals and some mobile robots use visual motion cues for object detection and navigation in structured surroundings<sup>1–4</sup>. Motion is commonly sensed by large arrays of small field movement detectors, each preferring motion in a particular direction<sup>5,6</sup>. Self-motion generates distinct 'optic flow fields' in the eyes that depend on the type and direction of the momentary locomotion (rotation, translation)<sup>7</sup>. To investigate how the optic flow is processed at the neuronal level, we recorded intracellularly from identified interneurons in the third visual neuropile of the blowfly<sup>8</sup>. The distribution of local motion tuning over their huge receptive fields was mapped in detail. The global structure of the resulting 'motion response fields' is remarkably similar to optic flow fields. Thus, the organization of the receptive fields of the so-called VS neurons<sup>9,10</sup> strongly suggests that each of these neurons specifically extracts the rotatory component of the optic flow around a particular horizontal axis. Other neurons are probably adapted to extract translatory flow components. This study shows how complex visual discrimination can be achieved by task-oriented preprocessing in single neurons.**

For several decades, coherent retinal image-shifts induced during locomotion have been considered a rich source of information about the momentary self-motion<sup>7</sup>. These retinal image-shifts, known as optic flow fields, can be described as vector fields where each vector indicates the direction and velocity of the local image-shift<sup>11,12</sup>. The structure of an optic flow field depends on the instantaneous self-motion<sup>12</sup>, which can be described in terms of its rotatory and translatory components. Electrophysiological<sup>13,14</sup> and behavioural experiments<sup>15</sup> in different animal species and psychophysical studies in humans<sup>3,16</sup> strongly suggest that optic flow is indeed exploited for the estimation of self-motion.

Globally, translatory and rotatory optic flow fields can be distinguished easily (compare Fig. 1a and b). However, in the first processing stages of the visual system, motion is analysed locally by elementary motion detectors (EMDs)<sup>5,6,17</sup>. Different kinds of self-motion may induce the same excitation in an EMD because the local flow vectors are quite similar or even equal at the respective position (see framed vectors on the right in Fig. 1a and b). Consequently, the two different self-motions cannot be distinguished at the level of the local EMD.

One simple way of overcoming these ambiguities is to integrate spatially the signals of those EMDs whose preferred directions correspond with the direction of the local velocity vectors of a particular optic flow field. In this case, the integrating neuron would respond best when the animal performs the corresponding self-motion in space. If this strategy were realized in the visual system, the integrating neurons should satisfy the following requirements: (1) the neurons should have extended receptive fields—because the ability to distinguish between different flow fields increases with increasing area of integration<sup>12</sup>; (2) the neurons' responses have to be motion-sensitive and direction-selective; (3) the local preferred direction of the neuron should depend in a characteristic way on the position of the local motion stimuli in the visual field.

The third visual neuropile (lobula plate) of the blowfly contains about 60 so-called tangential neurons that are individually identifiable from animal to animal<sup>18,9</sup>. Most of these neurons receive retinotopic input<sup>18</sup>, on their extended dendritic arborizations, from many local EMDs. Microsurgical and neurogenetic deletions

followed by behavioural experiments reveal that these neurons are involved in course control<sup>8,19–21</sup> and gaze stabilization<sup>22</sup>.

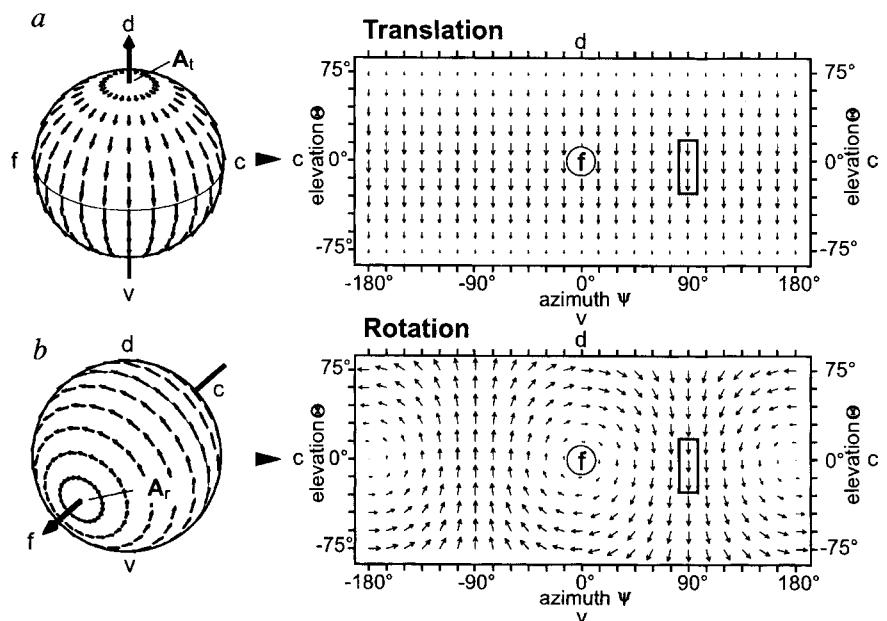
To decide whether or not the ten VS neurons—a distinct subgroup of the tangential neurons—are functionally specialized to process specific optic flow fields, we investigated their receptive fields in great detail. Using a fast stimulus procedure<sup>23</sup> (Fig. 2) during intracellular recordings from neurons in the right lobula plate, we determined their local preferred directions (LPDs) and local motion sensitivities (LMSs) at different positions in the visual field. The results were plotted as arrows on a map covering somewhat more than the right visual hemisphere where the angular position is defined by the azimuth  $\psi$  and the elevation  $\theta$ . The orientation of the arrows (Fig. 3, right) indicates the LPD and its length indicates the relative LMS normalized to the maximum response. The individual neurons could be recognized from animal to animal by intracellular dye injection and three-dimensional reconstruction<sup>24</sup>. For each of the ten VS-neurons the receptive field was mapped in at least six different animals.

Figure 3 shows the anatomy and the resulting response fields of the neurons VS1, VS6 and VS8 within the contours of the lobula plate. The first, striking impression of these response fields is their huge extent: they comprise most of the ipsilateral and some of the contralateral hemisphere. Second, all VS neurons respond to vertical downward motion but additionally to horizontal motion

(Fig. 3a–c) or even to vertical upward motion (Fig. 3a, c) in distinct regions of their receptive field. The most exciting result of this investigation, however, is the distribution of LPDs and LMSs within the response fields. In all cases the dorsal, equatorial and parts of the ventral response fields strongly resemble rotatory optic flow fields. This is most obvious for the response field of VS8: at about  $\psi = 45^\circ$  and  $\theta = -15^\circ$  the neuron is almost insensitive to visual motion (Fig. 3c), indicating the location of a flow-field singularity. In the whole response field of VS8, the local preferred directions are arranged tangentially around the motion axis, suggesting its specificity for rotation. The unequal distribution of local motion sensitivity corresponds qualitatively to the distribution of vector lengths in a rotatory flow field. It is zero at the singularities and maximum between the centres of rotation, for example at  $\psi = 135^\circ$  (Fig. 3c). The same global structure can be recognized in the response field of VS6 (Fig. 3b), where the axis of rotation corresponds with the body axis of the animal. In the response field of VS1 the rotatory structure is restricted to the frontal, dorsolateral and caudolateral regions of the visual field (Fig. 3a) and an estimated axis of rotation lies at about  $\psi = 90^\circ$ .

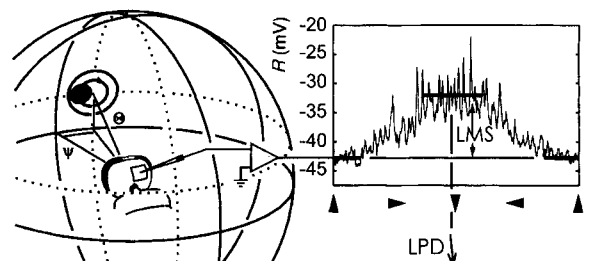
Generally, in the response fields of all ten VS neurons, the axes of rotation are almost aligned with the horizontal plane of the eye and the sensitivity to motion in the ventral part of the visual field is weaker than in the corresponding dorsal part (Fig. 3a–c). When

FIG. 1 Optic flow fields due to a, translation along the vertical and b, rotation around a horizontal axis, illustrated either by an external view of one visual hemisphere (left) or by a Mercator map of the whole visual space (right). Positions are specified by the angles of azimuth  $\psi$  and elevation  $\theta$ ;  $\psi < 0$  denotes the left,  $\psi > 0$  the right visual hemisphere (f represents frontal; c, caudal; d, dorsal, and v, ventral). The distribution of the velocity vectors depends on the momentary self-motion. Generally, there is no motion at the flow field singularities, which coincide with the motion axis; that is, the axis of translation  $A_t$  and the axis of rotation  $A_r$ . Maximum image-shifts are found midway between the singularities along the 'equator' of motion (thin great circles on the spheres). In translatory flow fields (a) local vectors are aligned radially along the meridians connecting the two singularities ( $A_t$  and the opposite singularity). In contrast, motion vectors in rotatory flow fields (b) are aligned tangentially around the singularities ( $A_r$  and the opposite singularity). The magnitude of the local velocity vectors induced by translations decreases with increasing distance to the objects of the environment, whereas flow vectors generated during rotations are invariant with respect to the distance. Globally, translatory and rotatory optic flow fields can be easily distinguished. However, because different types of self-motion can gen-



erate the very same local motion signals (frame at  $\psi = 90^\circ$ ), self-motion cannot be inferred unambiguously from local motion analysis. We show how this problem is solved in the fly's visual nervous system.

FIG. 2 Quick determination of the local preferred direction (LPD) and local motion sensitivity (LMS) of identified wide-field neurons in the third visual neuropile of the blowfly. A small black dot (diameter,  $7.6^\circ$ ) was moved at constant speed (2 cycles  $s^{-1}$ ) on a circular path (diameter,  $10.4^\circ$ ), thus travelling over only a minute part ( $<1\%$ ) of the fly's visual field. Great-circle lines indicate the visual field coordinates; dotted great-circle lines indicate the stimulus position (azimuth  $\psi$ , elevation  $\theta$ ). When the instantaneous direction of dot motion coincides with the LPD of the recorded neuron, it responds maximally by depolarization of its membrane potential ( $R$ ). The response delay causes a small phase shift of the response relative to the dot motion. This can be corrected by comparing the responses to clockwise and anti-clockwise dot motion. After correction the local motion tuning curves (right part) yield the LPD by their centre of gravity and the LMS by the amplitude difference between the quadrants of maximal and minimal responses (thick black lines).





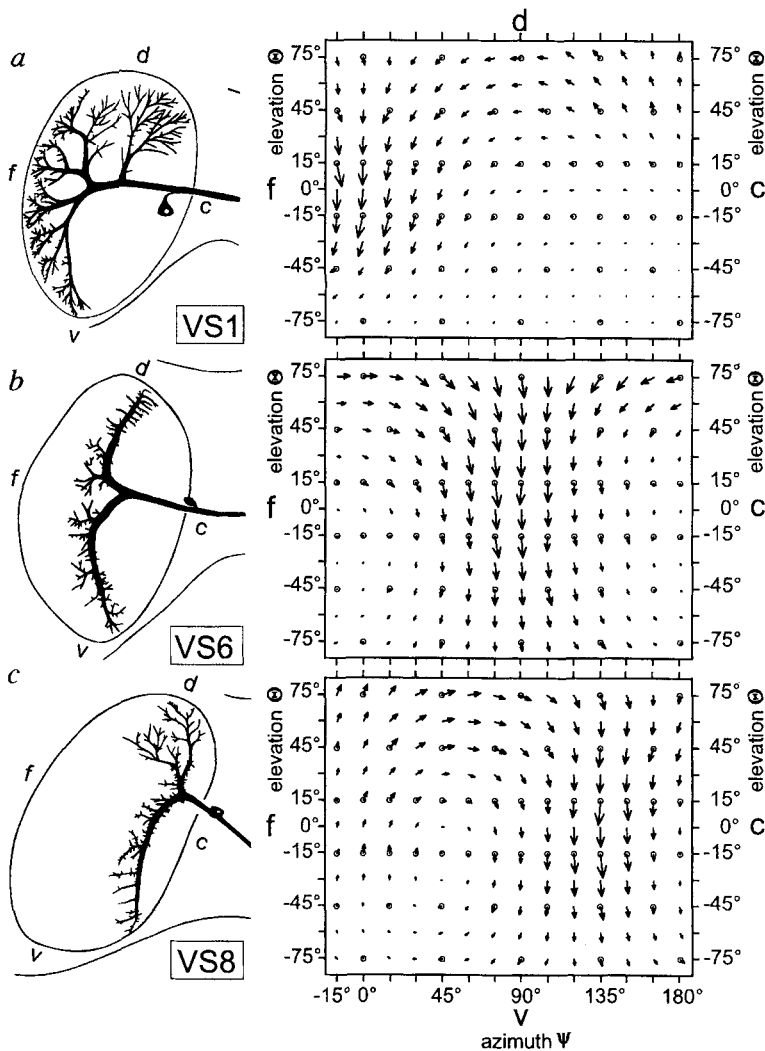
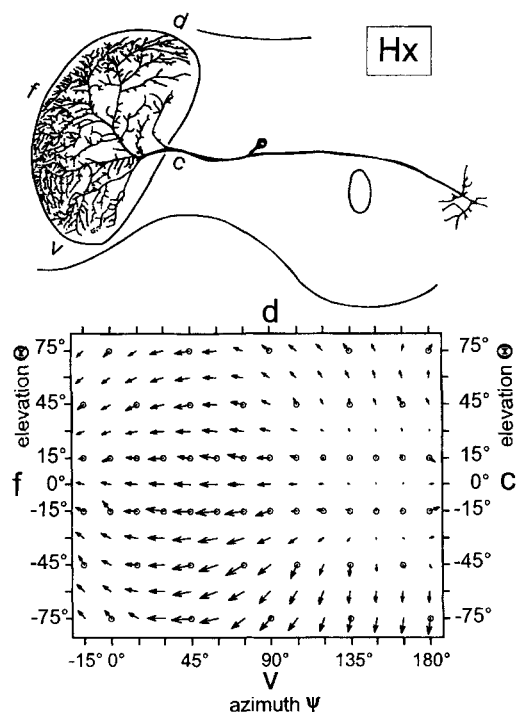


FIG. 3 a–c, Anatomy and response fields of the neurons VS1, VS6 and VS8. The local motion responses are shown in maps representing the right visual hemisphere ( $\psi > 0$ ) plus a vertical strip of the contralateral region of binocular overlap ( $\psi < 0$ ). Measurement positions are marked with small circles; the orientation of each arrow indicates the local preferred direction and its length the normalized local motion sensitivity. Arrows at other positions were interpolated. a, VS1 responds strongly to downward motion in the frontal and frontolateral area of the visual field. In the dorsolateral region it responds to horizontal back-to-front motion and in the dorsocaudal region even to upward motion. b, VS6 responds best to downward motion in the lateral visual field. In the dorso-frontal region it responds to horizontal front-to-back motion and in the dorsocaudal region to motion in the opposite direction. c, VS8 responds strongly to downward motion in the lateral visual field, but also to front-to-back motion in the dorsal frontolateral region. In the frontal visual field, the neuron responds to upward motion and in the ventral frontolateral field there is a weak but measurable response to horizontal back-to-front motion. The response fields of VS1, VS6 and VS8, and all other VS neurons<sup>26</sup>, are very similar to rotatory optic flow fields with roughly horizontal axes oriented at different angles of azimuth (compare Fig. 1b). Compared with optic flow fields, the sensitivity of all VS neurons is reduced in the ventral parts of the visual field.

FIG. 4 Anatomy and response field of the Hx neuron. Like the VS neurons, Hx is sensitive to motion over large parts of the visual field. However, in contrast to the VS neurons, Hx is highly sensitive to horizontal back-to-front motion in the frontolateral visual field ( $\psi = 45^\circ$ ). At  $\psi = 135^\circ$  and  $\Theta = 0^\circ$  the neuron does not respond to visual stimulation. Around this spot of low sensitivity the preferred directions are oriented radially. Thus, the structure of the Hx response field is remarkably similar to a translatory optic flow field with a singularity at  $\psi = 135^\circ$  and  $\Theta = 0^\circ$ . This spiking neuron conveys motion information via a thin axon from one lobula plate to the contralateral protocerebrum. In contrast to the VS neurons, Hx is more sensitive to motion in the ventral than in the dorsal part of the visual field.



mappings are repeated in a long recording in one fly, the scatter of axis positions around the mean is less than  $\pm 2^\circ$  (standard deviation, s.d.). For each of the ten groups of VS neurons, recorded in different flies, the scatter ranges from  $\pm 6^\circ$  to  $\pm 20^\circ$  (s.d.), with a mean value of less than  $\pm 12^\circ$  (s.d.).

The global structure of the receptive field organization is not at all the same in all lobula plate neurons. Figure 4 shows the neuron 'Hx' that does not belong to the group of VS neurons and has not yet been described. Its response field also has a region of very low sensitivity ( $\psi = 135^\circ$ ,  $\theta = 0^\circ$ ). In this case, however, the LPDs are not oriented tangentially but radially with respect to the spot of low motion sensitivity. Thus, the global structure of this response field resembles a translatory optic flow field—with a centre of translation at about  $\psi = 135^\circ$  corresponding to motion within the horizontal plane in the caudolateral direction. Because the tangential neurons are inhibited by motion in the null direction the spiking activity of Hx is suppressed during straight-ahead translation. Increasing deviations from this course would cause an increasing disinhibition and thus higher spiking activity of the neurons. The sensitivity distribution qualitatively matches the distribution of the amounts of the local velocity vectors in a translatory optic flow field, that is, the strongest responses were measured about  $90^\circ$  away from the centre of translation (Fig. 4,  $\psi = 45^\circ$ ). In contrast to VS neurons, Hx is more sensitive to motion in the ventral than in the corresponding dorsal parts of the visual hemisphere.

The global structure of the response fields strongly suggests that different tangential neurons in the fly's visual system act as sensory filters which extract specific components of self-motion from the momentary optic flow. Some neurons (for example, Hx; Fig. 4) extract a translatory component, whereas others sense rotatory components—in the case of the VS neurons rotations around horizontal axes with different azimuthal orientations. This interpretation of the VS neurons' function is supported by three independent experimental findings: (1) in *Drosophila*, these neurons are essential for the generation of compensatory head rotations<sup>20,25</sup>; (2) in *Calliphora* the response of the neurons increases with pattern size until they gradually saturate<sup>10</sup>; (3) the neuron H1 (ref. 8) in *Calliphora stygia* responds best when the local motions in a composite stimulus coincided with the respective local preferred directions<sup>26</sup>. Therefore it must be expected that every VS neuron is maximally excited when it is stimulated over the entirety of its receptive field with the locally optimal motion stimuli, that is, when it 'sees' the optic flow field to which it is tuned. Interestingly, both types of filter neurons seem to be adapted to the anisotropic 'richness' of the optic flow experienced by an insect during flight over structured ground. The 'translation sensor' (Hx) has a high motion sensitivity in the ventral part of its receptive field, where strong translation signals are to be expected during flight at a finite height. The 'rotation sensors' (VS neurons; Fig. 3) have a low motion sensitivity in the ventral visual field, which automatically reduces crosstalk from translation signals into rotation channels. Their high sensitivity around and above the horizon (Fig. 3) exploits the large distances to the horizon and clouds to preferentially sense rotations, because translatory flow components decrease with distance, whereas rotatory components do not.

A quantitative evaluation<sup>27</sup> of the different axes of rotations showed that the VS neurons can be subdivided into three functional groups. The first group (VS1–VS3) is adapted to sense rotatory self-motions around the transverse body axis (pitch-rotation). The second group (VS4–VS7) appears to be specialized to signal rotations around the longitudinal body axis (roll-rotation). The axes of rotation of the third group (VS8–VS10) lie more or less between the main body axis and the transverse body

axis. If the signals of the VS neurons are combined in the appropriate way, the relative level of activity within the three groups is sufficient to control body rotations around any horizontal axis of rotation<sup>28</sup>. □

## Methods

**Preparation.** Female flies (*Calliphora erythrocephala* Meig.) aged 1–3 d were briefly anaesthetized with CO<sub>2</sub> and fixed with wax at the centre of a spherical gimbal that allowed us to place a small field stimulator almost anywhere in the visual field<sup>23</sup>. The optic lobe was exposed by bending the fly's thorax ventrad, cutting a small window into the back of the head capsule, and teasing air sacs and tracheae aside. For details, see ref. 29.

**Electrophysiology.** Neurons were impaled, recorded and stained intracellularly with glass micropipettes (puller: Sutter Instruments, P 87) filled with 3% Lucifer Yellow CH in 1 M LiCl and with an input resistance of 40–60 M $\Omega$ . The neuronal signals were recorded with a high-impedance voltage follower (MPI, TK88), stored on a digital audio tape (Bio-Logic, DTR 1800), sampled at 0.7 kHz via an analogue-to-digital converter (Data Translation, DT 2801A) into a personal computer (IBM, PC 486), and processed with programs written in ASYST.

**Histology.** Fly brains were prefixed *in situ*, embedded in Spurr's epoxy medium<sup>30</sup>, frontally sectioned at 10  $\mu$ m, and inspected with a fluorescence microscope (Zeiss, Axiophot, FITC). Neurons were identified either by camera lucida reconstructions from colour transparencies (Kodak, Elite 200) or by stereograms computed from three-dimensional reconstructions<sup>24</sup>.

**Determination and mapping of local response properties.** The local preferred directions (LPD) and local motion sensitivities (LMS) of each neuron were determined at 48 positions in the right visual hemisphere and four positions in the area of binocular overlap ( $\psi = -15^\circ$ , Fig. 3); the left eye was occluded with black paint. A brief description of this new method is given in Fig. 2 and is available as Supplementary Information. The method and its performance will be described in detail elsewhere<sup>23</sup>.

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# Neurturin, a relative of glial-cell-line-derived neurotrophic factor

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THE normal development of the vertebrate nervous system entails the death of 30–70% of the neurons originally generated in most neuronal populations<sup>1</sup>. This naturally occurring cell death is regulated by specific neurotrophic factors that promote neuronal survival and which are produced in limiting quantities by target cells, glial cells and neurons. These factors are also of potential utility as therapeutic agents for neurodegenerative diseases<sup>2</sup>. Here we describe the purification and cloning of a new neurotrophic factor, identified on the basis of its ability to support the survival of sympathetic neurons in culture. This factor, neurturin, is structurally related to glial-cell-line-derived neurotrophic factor (GDNF)<sup>3</sup>. These factors can each activate the MAP kinase signalling pathway in cultured sympathetic neurons and support the survival of sympathetic neurons, as well as of sensory neurons of the nodose and dorsal root ganglia. Thus, neurturin and GDNF together now define a new family of neurotrophic factors.

Sympathetic neurons obtained from the superior cervical ganglia (SCG) of neonatal rats can be maintained in culture in the presence of nerve growth factor (NGF) and undergo apoptosis when deprived of NGF<sup>4,5</sup>. We investigated whether factors other than NGF can promote sympathetic neuron survival in culture, because the identification of such factors should provide insight into the development and maintenance of the nervous system. While developing a mammalian cell expression system for production of recombinant growth factors<sup>6</sup>, we discovered that the conditioned medium of Chinese hamster ovary (CHO) cells contained an activity that could support the long-term survival of superior cervical ganglion (SCG) sympathetic neurons in culture (Fig. 1). This SCG survival-promoting activity was thermo-

stable, sensitive to trypsin and had a relative molecular mass ( $M_r$ ) of >10,000 (10K) in dialysis and ultrafiltration experiments. This activity, apparently attributable to a peptide factor, was not affected by neutralizing antibodies against NGF, and its chemical properties distinguished it from LIF and CNTF, the only other peptide factors then known to promote neuronal survival in this assay<sup>7</sup>.

The CHO-derived SCG-survival-promoting activity was purified ~400,000 fold by using a combination of heparin affinity, copper affinity and ion-exchange chromatography. A single protein ( $M_r$  25K on non-reducing SDS-PAGE) copurified with the survival-promoting activity (Fig. 2a,b). Furthermore, survival-promoting activity was eluted from a gel slice corresponding to the region where this 25 K protein migrated on SDS-PAGE (results not shown). We termed this purified 25K protein neurturin, and found that it promoted the survival of 100% of neurons supported by NGF at 750 pg ml<sup>-1</sup> (30 pM) (Fig. 2c); the half-maximal effective dose ( $EC_{50}$ ) of neurturin in this assay was 425 pg ml<sup>-1</sup> (17 pM).

Amino-terminal and internal partial amino-acid sequencing of neurturin indicated that it was a new factor; these sequences were used to isolate mouse complementary DNA and genomic clones by degenerate polymerase chain reaction (PCR) (Fig. 3a). Mouse neurturin cDNA contains an open reading frame encoding a 195-amino-acid protein. This 195-residue protein is predicted to contain a 19-amino-acid signal sequence<sup>8</sup> and 76-amino-acid pro-region. Proteolytic cleavage of the pro-protein at an RXXR consensus sequence<sup>9</sup> should yield a 100-residue mature protein with an N terminus corresponding to that of the purified Chinese hamster protein.

Human neurturin genomic clones were isolated on the basis of their high sequence similarity to the mouse clones. The inferred mature human neurturin protein is 91% identical to mature mouse neurturin, but is predicted to have an N-terminal extension of two amino acids inserted immediately after the conserved RXXR cleavage site. The mature mouse and human neurturin proteins have a predicted  $M_r$  of 11.5 K, about half that of the purified protein, indicating that the purified protein could be a homodimer.

The amino-acid sequence of mature neurturin shares about 42% similarity with mature GDNF (Fig. 3b), which promotes the survival of dopaminergic neurons in embryonic midbrain cultures<sup>3</sup>. Sequence alignments suggest that neurturin, like GDNF, is also a distant member of the transforming growth factor (TGF)- $\beta$  superfamily. This superfamily comprises more than 25 proteins with a wide array of biological activities<sup>10</sup>. The amino-acid sequence similarity of neurturin and of GDNF to other TGF- $\beta$

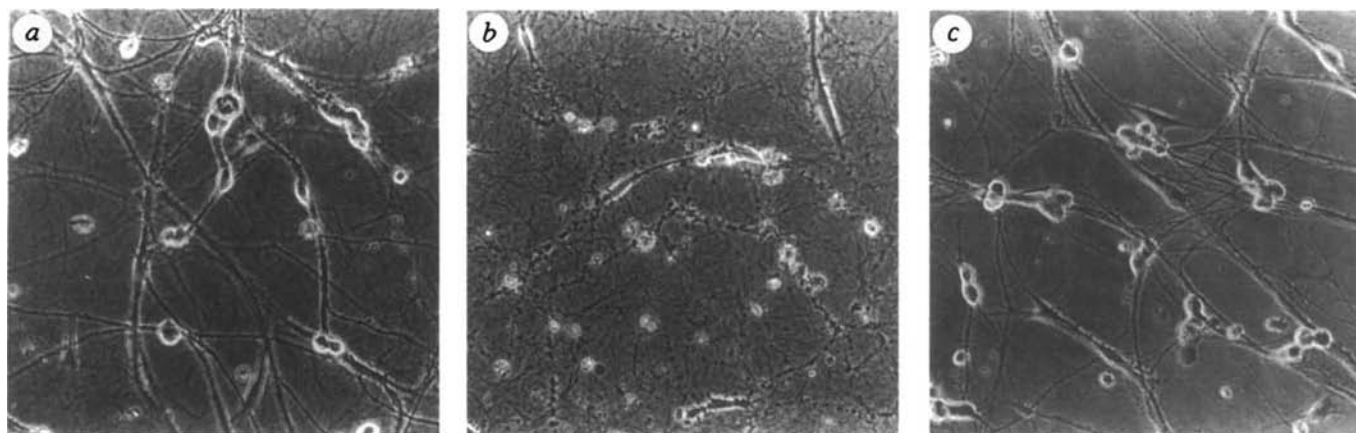


FIG. 1 Promotion of sympathetic neuronal survival *in vitro* by neurturin. Sympathetic neurons were obtained from the superior cervical ganglia of E21 rats and cultured for 5 days in the presence of NGF. On day 5, the culture medium was either maintained as before (a) or switched to medium

containing b, anti-NGF<sup>4</sup>, or c, anti-NGF plus partially purified neurturin. Cultures were photographed 2 d after treatment. Survival was enhanced for at least 8 d after addition of anti-NGF in the presence of neurturin (not shown).



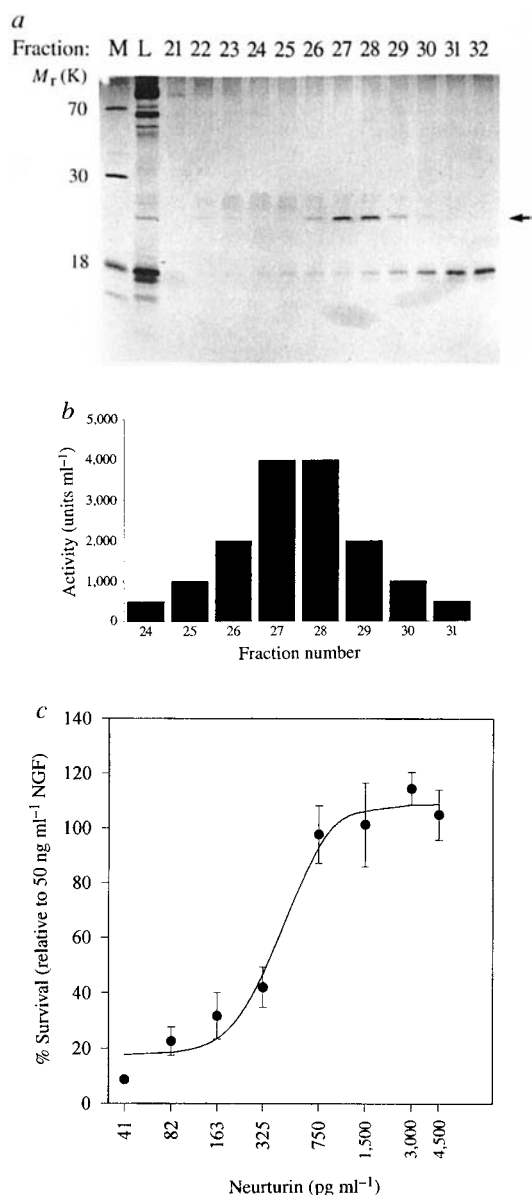


FIG. 2 Co-purification of the sympathetic survival-promoting activity with a 25K protein. **a**, Fractions obtained from the final Mono-S column in the purification of neurturin were analysed, without reduction by electrophoresis on SDS-PAGE and silver staining. Arrow, 25K neurturin. **b**, The same Mono-S fractions were screened for survival-promoting activity in the SCG survival assay. The units in a fraction were determined by using a range of concentrations in the SCG assay: one survival unit was defined as the minimum volume promoting maximum neuronal survival. **c**, Concentration-response curve of sympathetic survival promotion by neurturin. SCG cultures containing 300 neurons per well in 24-well plates were treated on day 5 with anti-NGF and purified neurturin. The mass of purified neurturin added to the cultures was estimated by amino-acid analysis. Bars represent standard error of the mean.

FIG. 4 Neurturin mRNA expression. **a**, Northern blot analysis, in which neurturin mRNA (arrow) was detected by hybridization with a 588-bp DNA probe containing the entire mouse coding sequence, after agarose gel electrophoresis of 40  $\mu$ g total RNA from the indicated tissues and transfer to a nylon membrane. Besides hybridizing to the 1.0-kb band, the neurturin probe also detected an unidentified ~200-nucleotide transcript (lower band) which could not encode a full-length neurturin protein. **b**, RT-PCR analysis. Relative levels of neurturin mRNA were determined in different rat tissues by semi-quantitative RT-PCR using primers synthesized from the neurturin cDNA sequence. Perfusion of animals with PBS to reduce neurturin mRNA in their blood had no effect on the levels in other tissues, including brain (results not shown).

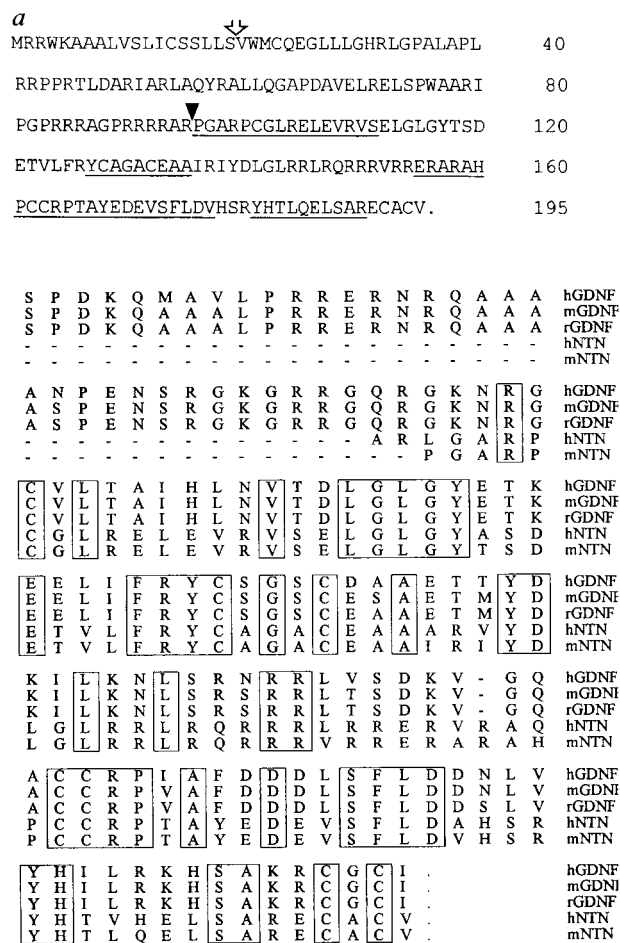
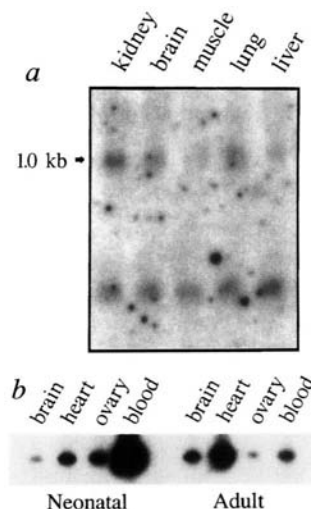


FIG. 3 **a**, Amino-acid sequence of mouse neurturin. The open arrow indicates the predicted cleavage site of a putative signal sequence; the arrowhead indicates the predicted cleavage site in the pro-protein that generates mature neurturin. Sequences corresponding to Chinese hamster neurturin are underlined. The human and mouse neurturin cDNA sequences have been deposited in Genbank (accession numbers U78109 (mouse) and U78110 (human)). **b**, Alignment of inferred mature protein sequences for mouse and human neurturin, and mouse, rat and human GDNF. Sequences were aligned using the Clustal method<sup>30</sup> of multiple sequence alignment in Lasergene sequence-analysis software. (DNASTAR, Madison, WI)



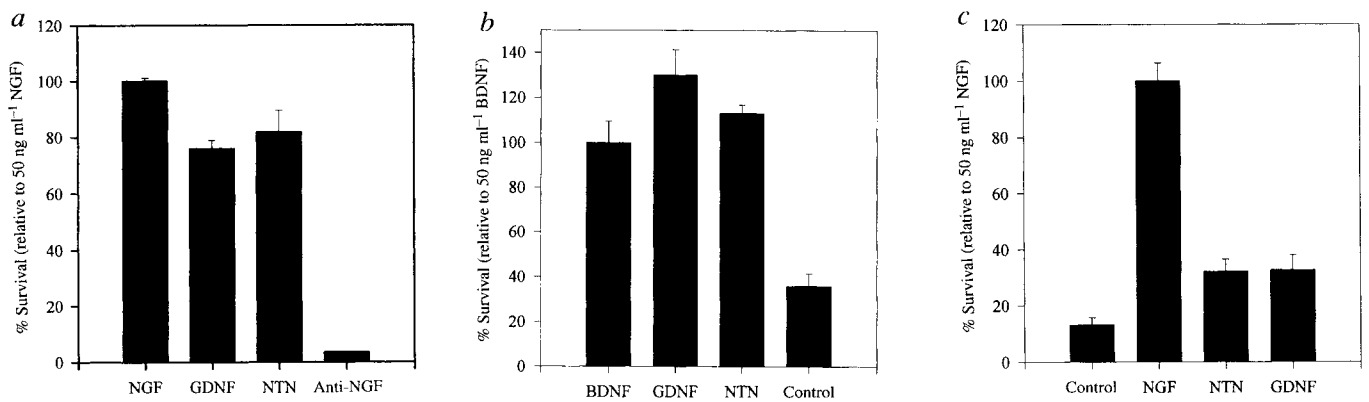


FIG. 5 Quantification of the survival of *a*, SCG neurons; *b*, NG neurons; and *c*, DRG neurons in the presence of neurturin (NTN), GDNF, and neurotrophins. *a*, E21 SCG neurons were cultured for 2 d in the presence of NGF before addition of anti-NGF plus the indicated factors and culture for 2 more days. *b*, E18 NG neurons were cultured for 1 day in the presence of BDNF before removal of BDNF and addition of the factors indicated for 2

more days of culture. *c*, E15 DRG neurons were cultured for 3 days in the presence of the indicated factors. NGF, BDNF or GDNF were added at 50 ng ml<sup>-1</sup>; purified neurturin was present at 6 ng ml<sup>-1</sup>. After treatment, cultures were fixed and stained, and the surviving neurons counted. Error bars represent standard errors of the means.

family members is less than 20%, and is based primarily on the seven conserved cysteine residues found in the same relative spacing across the entire family. The high similarity of neurturin to GDNF relative to the other TGF- $\beta$  family members establishes a new subfamily of peptide growth factors, underscored by their shared neurotrophic activity. The isolation of other, as-yet unidentified members should now be feasible by methods based on amino-acid sequences conserved between neurturin and GDNF.

The expression of neurturin in different murine tissues was examined by RNA blot analysis: a ~1.0-kilobase (kb) messenger RNA was detected in varying amounts (Fig. 4*a*). The size of this transcript is consistent with that predicted from mouse complementary DNAs isolated by the RACE technique<sup>11</sup> that contain a 350-nucleotide 5' untranslated region and a 75-nucleotide 3' untranslated region, in addition to the 588-base-pair (bp) coding region.

The expression of neurturin mRNA was analysed by PCR with reverse transcription (RT-PCR). Expression was widespread in neonatal and adult rat tissues (Fig. 4*b*). Expression in both the neonatal and adult brain indicates that neurturin may regulate development and maintenance of the central nervous system. Neurturin expression was high in neonatal blood, suggesting that neurturin could be distributed in the circulation. Its widespread distribution suggests that it might control the size of non-neuronal cell populations such as haemopoietic cells.

To demonstrate that neurturin cDNA directs the synthesis of biologically active neurturin, the mouse neurturin-coding sequence was inserted downstream of the cytomegalovirus (CMV) immediate-early promoter in a pCB6 expression vector<sup>12</sup> and transiently transfected into NIH3T3 cells. Conditioned medium from NIH3T3 cells transfected with either control plasmid or the neurturin expression vector (pCMV-NTN) was assayed by direct addition to SCG cultures treated with neutralizing antisera to NGF. Conditioned medium from cells transfected with pCMV-NTN contained survival-promoting activity in the sympathetic neuronal survival assay that was comparable to that of

NGF (data not shown), indicating that neurturin cDNA encodes the active protein. Active neurturin has also been obtained by expression of the mature coding region in *Escherichia coli* (R.O.H., P.T.K., P.A.L., E.M.J., and J.M., unpublished results).

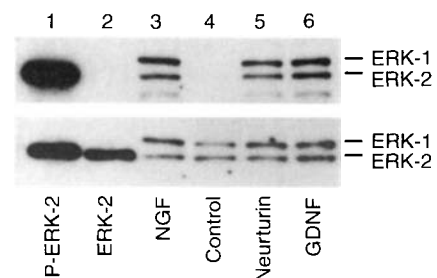
The biological activity of neurturin and GDNF was compared in *in vitro* neuronal-survival assays. Neurturin and GDNF both promote survival of nodose ganglia (NG) sensory neurons and a small population of dorsal root ganglia (DRG) sensory neurons, in addition to SCG sympathetic neurons (Fig. 5). The similarity in their activities and sequences suggests that neurturin and GDNF may act through common signalling pathways. We therefore tested their effects on MAP kinase activation in sympathetic neurons, as this pathway is activated by the trophic effects of NGF<sup>13</sup>. An antibody specific for phosphorylated MAP kinase revealed that, like NGF, both neurturin and GDNF activated the ERK-1 and ERK-2 isoforms of MAP kinase in sympathetic neurons (Fig. 6), indicating that these factors use similar signalling in sympathetic neurons.

GDNF promotes the survival of several populations of neurons<sup>14</sup>, including motor<sup>15-18</sup> and midbrain dopaminergic neurons<sup>19-23</sup>. The parallel biological activities of GDNF and neurturin described here may extend to motor neurons and dopaminergic neurons as well, suggesting that neurturin as well as GDNF may be of value in the treatment of Parkinson's and motor neuron diseases. The activity of GDNF is mediated by the Ret receptor tyrosine kinase in association with the glycosyl-phosphatidylinositol-linked protein GDNFR- $\alpha$  (refs 24-27). Neurturin may also act through these or related proteins. □

## Methods

**Primary neuronal cultures.** Dissociated primary cultures of E21 rat SCG neurons were prepared from Sprague Dawley rats (Harlan) as described<sup>7</sup>, except that dissociated cells were pre-plated for 2 h on a 100-mm Falcon culture dish to remove non-neuronal cells, before plating in a 100- $\mu$ l drop in 24-

FIG. 6 Neurturin and GDNF activate the ERK-1 and ERK-2 isoforms of MAP kinase (MAPK) in sympathetic neurons. Six-day-old cultures of SCG neurons were deprived of NGF for 12 h then treated with neurturin, GDNF or NGF. MAPK activation was determined by probing western blots with a phospho-specific MAPK antibody (top). The same blot was then stripped and reprobed with a control MAPK antibody that recognizes both phosphorylated and non-phosphorylated forms of ERK-1 and ERK-2 (bottom). Lane 1, 2 ng phosphorylated ERK-2 protein (P-ERK-2); lane 2, 2 ng non-phosphorylated ERK-2; lanes 3-6, lysate from sympathetic neurons treated with 50 ng ml<sup>-1</sup> NGF, no factor (control), 50 ng ml<sup>-1</sup> neurturin or 50 ng ml<sup>-1</sup> GDNF, as indicated.



well tissue culture plates containing a double layer of rat-tail collagen, prepared as described<sup>28</sup>. The cells were maintained for up to 5–6 days at 37 °C in 5% CO<sub>2</sub> before assay. Cultures contained ~1,200 neurons per well, except where indicated. Primary dissociated cultures of neurons from the nodose ganglia (NG) and dorsal root ganglia (DRG) were prepared from embryos as for SCG cultures.

**Purification and cloning of neurturin.** For details of the purification and molecular cloning of neurturin, see Supplementary Information. Briefly, serum-free conditioned medium from hSB CHO cells<sup>6</sup> (CHO-CM) was applied in 25 l batches to a 100 ml heparin agarose column and survival-promoting activity eluted in 25 mM HEPES pH 7.4, 1.0 M NaCl. The eluates from two heparin agarose columns were combined and diluted to 0.5 M NaCl and then applied to a 16 ml SP Sepharose HP column. Survival-promoting activity was eluted in 1.0 M NaCl in 25 mM HEPES, pH 7.4, with 0.02% Tween 20, and applied to a Chelating Sepharose HR 10/2 column (Pharmacia Biotech) charged with CuSO<sub>4</sub> and the column was developed in a 40 ml linear gradient of 0 to 300 mM glycine in 25 mM HEPES, pH 7.4, 1.0 M NaCl, 0.02% Tween 20 using a Pharmacia FPLC system. Fractions containing the peak of survival-promoting activity were pooled, diluted to 0.45 M NaCl and applied to a Mono S HR 5/5 column (Pharmacia Biotech). The column was eluted with a 35 ml linear gradient of 0.45 M to 1.0 M NaCl in 25 mM HEPES, pH 7.4, 0.02% Tween 20. Column fractions containing the peak of survival-promoting activity were identified by addition of aliquots to the SCG assay at a range of concentrations. N-terminal sequencing of purified neurturin yielded sequence SGARPXGLRE-LEVSVS. For internal sequencing, 50 l CHO-conditioned medium were purified in the first three chromatography steps (see Supplementary Information), with the Cu<sup>2+</sup>-chelating Superose column being eluted with a gradient of 0–60 mM glycine (4 ml), 60 mM glycine (10 ml), 60–300 mM glycine (32 ml); purified neurturin was isolated in an acrylamide-gel slice and proteolytic fragments were purified after digestion *in situ* with endoproteinase lys-C or trypsin. The three internal amino-acid sequences obtained were XXVEAKPCGPTAYEDXVSFLSV (from lys-C digestion); XCAGAXEAAV; and YHTLQEL SAR (from trypsin digestion). Partial cDNA fragments were amplified using degenerate primers corresponding to the amino-acid sequences. Genomic clones were obtained by PCR screening of mouse and human genomic libraries in the bacteriophage P1 vector<sup>29</sup> (Genome Systems). The presumed full-length mouse neurturin cDNA was identified by the rapid amplification of cDNA ends (RACE)<sup>11</sup> with adaptor-ligated E21 brain cDNA prepared using a Marathon RACE kit (Clontech). The 588-bp coding sequence of neurturin mRNA was amplified from mouse post-natal-day-1 brain cDNA, with primers corresponding to the first 7 codons (5'-GCGACGCGTACCATGAGGCGCTGGAAGGCAGCGCCCTG-3' and the last 5 codons (5'-GACGGATCCGCATCACGCACGCCACTC-3') of neurturin.

**RT-PCR.** Primers (5'-TAGCGGTGTGTACGTCCAGGAAGGACCTCGT-3') and (5'-CAGCGACGACGCGTGCAGAAAGAGCG-3') were used for amplification of neurturin cDNA. The amount of template cDNA used in each reaction was normalized to the amount of actin mRNA, as determined by RT-PCR with primers (5'-TCGGAGACGGGGTACCA-3') and (5'-GTCCAGACGAGGATGGCAT-3'). Amplification of template cDNA was in the linear range for the number of PCR cycles (26) and the amount of poly(A)<sup>+</sup> RNA template used (< 50 ng for each tissue).

**NIH3T3 transfections.** See Supplementary Information for figure. NIH3T3 cells were plated at a density of 400,000 cells per well (34.6 mm diameter) in 6-well plates 24 h before transfection using lipofectamine (Gibco BRL) according to the manufacturer's instructions. 24 h after addition of DNA-liposome complexes, the medium was replaced with 1 ml DME medium containing 10% calf serum and 25 µg ml<sup>-1</sup> heparin. Cells were incubated for 24 h before the conditioned medium was collected. Conditioned medium (250 µl) from cells transfected with either pCMV-NTN or control pCB6 was mixed with 250 µl SCG culture medium containing anti-NGF and added to 5-day-old SCG cultures prepared from neonatal rats. 48 h after addition of conditioned medium, the cultures were fixed in 4% paraformaldehyde, stained with crystal violet, and the surviving neurons counted.

**MAP kinase assay.** After treatments, cultured neurons were lysed directly in Laemmli sample buffer, boiled for 5 min, run on SDS-PAGE, and transferred to PVDF membranes. ERK-1 and ERK-2 proteins were detected using a PhosphoPlus MAPK antibody kit (New England Biolabs) according to the manufacturer's instructions.

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## Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis

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**THE D-type cyclins (D1, D2 and D3) are critical governors of the cell-cycle clock apparatus during the G1 phase of the mammalian cell cycle. These three D-type cyclins are expressed in overlapping, apparently redundant fashion in the proliferating tissues<sup>1,2</sup>. To investigate why mammalian cells need three distinct D-type cyclins, we have generated mice bearing a disrupted cyclin D2 gene by using gene targeting in embryonic stem cells. Cyclin D2-deficient females are sterile owing to the inability of ovarian granulosa cells to proliferate normally in response to follicle-stimulating hormone (FSH), whereas mutant males display hypoplastic testes. In ovarian granulosa cells, cyclin D2 is specifically induced by FSH via a cyclic-AMP-dependent pathway, indicating that expression of the various D-type cyclins is under control of distinct intracellular signalling pathways. The hypoplasia seen in cyclin D2<sup>-/-</sup> ovaries and testes prompted us to examine human cancers deriving from corresponding tissues. We find that some human ovarian and testicular tumours contain high levels of cyclin D2 messenger RNA.**

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TABLE 1 Sterility of cyclin D2-deficient females

|             | Control         | Cyclin D2 <sup>-/-</sup> |
|-------------|-----------------|--------------------------|
| Pregnancies | 6/9*            | 0/109*                   |
| Blastocysts | 10/14†<br>(60)‡ | 1/13†<br>(7)‡            |
| Oocytes     | 6/6§<br>(215)   | 0/8§<br>(0)              |

Sexually mature, control (wild-type or cyclin D2<sup>-/-</sup>) and cyclin D2<sup>-/-</sup> females were mated with wild-type males.

\*The ratio of pregnancies to matings (the occurrence of mating was scored by the presence of a vaginal plug).

†The proportion of females that contained blastocysts in their uteri 3.5 days after mating.

‡The total number of observed blastocysts.

§The proportion of females that released oocytes following super-ovulation treatment<sup>5</sup>.

|| Total number of released oocytes following superovulation treatment<sup>5</sup>.

Cyclin D2<sup>-/-</sup> mice, derived by breeding D2<sup>+/-</sup> heterozygotes, were born at the expected mendelian ratio. Despite widespread expression of cyclin D2 during normal mouse embryo development (Fig. 1a, and results not shown), cyclin D2<sup>-/-</sup> animals were indistinguishable phenotypically from their wild-type littermates. The lack of developmental defects in D2<sup>-/-</sup> mice echoes the circumscribed phenotype of cyclin D1<sup>-/-</sup> mice<sup>3,4</sup>. It is probably attributable to the fact that most tissues of the developing embryo express more than one D-type cyclins, which we presume function in a redundant fashion.

A clear abnormality of cyclin D2<sup>-/-</sup> mice became apparent, however, when we mated these animals and found the mutant females to be infertile (Table 1). Furthermore, no oocytes were released by D2<sup>-/-</sup> females when they were subjected to a super-ovulation regimen known to force ovulation<sup>5</sup> (Table 1), pointing to ovarian failure as the primary cause of infertility.

Although the number of ovarian follicles and oocytes was normal in mutant females (not shown), the number of somatic granulosa cells surrounding each oocyte was reduced when compared with those in wild-type animals. This difference was first noticeable at about 8 days after birth. By 5 weeks of age, normal wild-type ovaries contained large follicles composed of up to ten layers of granulosa cells surrounding the oocytes; the follicles of the D2<sup>-/-</sup> ovaries, in contrast, were much smaller and rarely contained more than four layers of granulosa cells (Fig. 2a, b). The ovaries of heterozygous females were indistinguishable from those of wild-type mice (not shown).

We compared the responses of wild-type and mutant ovaries to FSH, which normally stimulates granulosa cell proliferation<sup>6</sup>. As expected, administration of FSH to wild-type females led to a rapid proliferation of the granulosa cell layer within 48 hours of injection (compare Fig. 2a and c). In contrast, the proliferative response of granulosa cells in the D2<sup>-/-</sup> ovaries was minimal (Fig. 2b, d).

When FSH treatment was followed by injection of luteinizing hormone (LH), wild-type follicles underwent ovulation and their granulosa cells assumed a terminally differentiated state, enabling the follicles to develop into corpora lutea (Fig. 2e, g). The hypoplastic follicles of D2<sup>-/-</sup> mice, however, failed to release oocytes and instead proceeded to form corpora lutea carrying trapped oocytes (Fig. 2f, h). This defect could not be attributed to impaired signalling by the LH receptors, because the prostaglandin endoperoxide synthase-2 and progesterone receptor mRNAs were normally induced in the mutant granulosa cells following LH treatment (not shown).

The oocytes trapped in the follicles of D2<sup>-/-</sup> mice were apparently normal. Thus, when manually liberated from surrounding granulosa cells and subjected to *in vitro* maturation<sup>7</sup>,

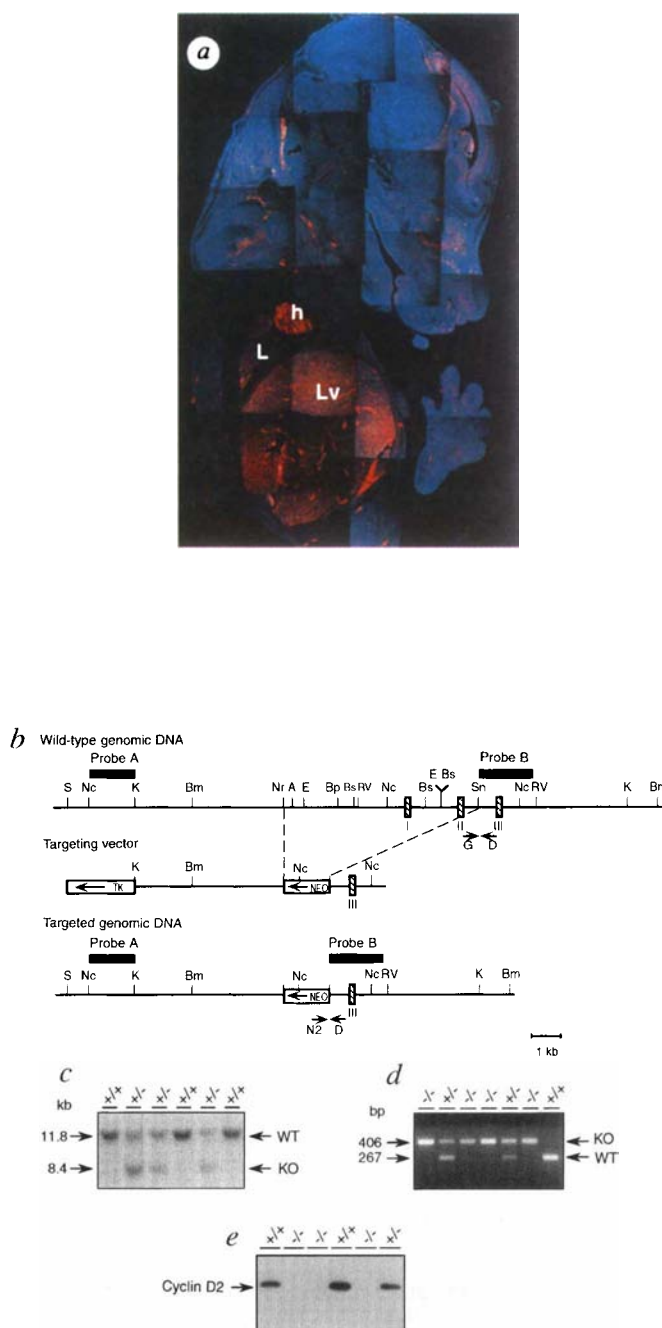


FIG. 1 Targeted inactivation of the mouse cyclin D2 gene. a, Expression of cyclin D2 mRNA during normal mouse embryogenesis detected by *in situ* hybridization. Composite photomicrographs of a sagittal section of a 14.5-d post-coitum embryo. Lv, liver; L, lung; h, heart. Original magnification,  $\sim 15\times$ . b, Cyclin D2 gene targeting strategy. The coding exons are shown as striped boxes; only the positions of coding exons I, II and III were mapped. The distal 5 kb of the genomic fragment shown have not been fully mapped and may contain additional restriction sites. Probes A and B used for Southern blotting analysis, as well as PCR primers G, D and N2, are indicated. NEO, the neomycin phosphotransferase gene; TK, thymidine kinase gene (arrows indicate the transcriptional orientation of these genes); S or Sn, *Sna*BI; Nc, *Nco*I; K, *Kpn*II; Bm, *Bsm*II; Nr, *Nru*I; A, *Aat*II; E, *Eag*I; Bp, *Bsp*EI; Bs, *Bss*III; RV, *Eco*RV. c, Southern blot analysis of genomic DNA extracted from ES cell clones and hybridized with probe A. d, PCR amplification products of DNA extracted from mouse tails. The sizes of wild type (WT) and mutant (KO) alleles are indicated in c and d. e, Immunoblot analysis of mouse embryo lysates probed with anti-cyclin D2 antibody.



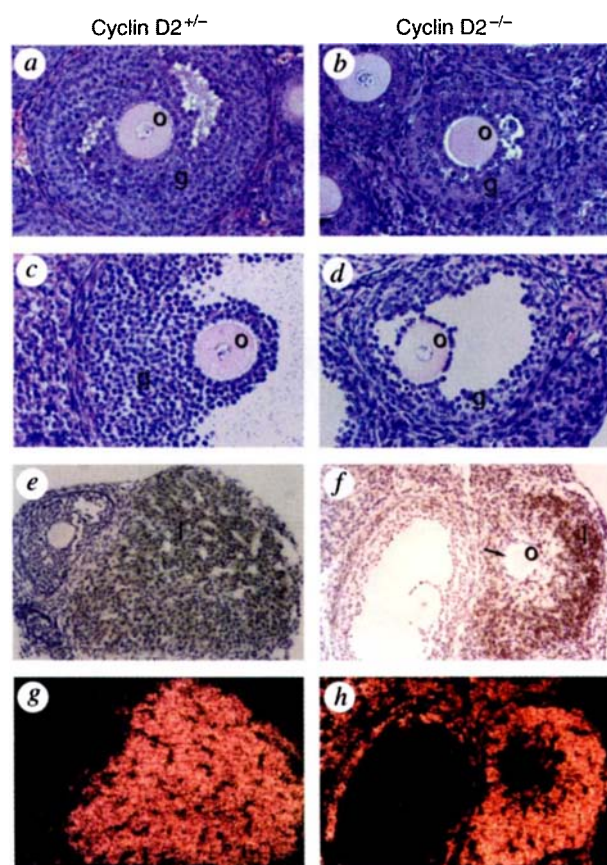


FIG. 2 Impaired ovarian granulosa cell proliferation in cyclin D2<sup>-/-</sup> mice. Sections of ovaries derived from 3-week-old cyclin D2<sup>-/-</sup> (a, c, e, g) or cyclin D2<sup>+/+</sup> (b, d, f, h) females. a and b, No hormones administered; haematoxylin and eosin staining; c and d, 45 h after FSH injection, same staining; e and f, 48 h after FSH injection, animals received LH injection and were killed 48 h later. *In situ* hybridization localization of the mRNA encoding the cytochrome-P450 cholesterol-side-chain cleavage (P450scc) enzyme (dark signal) was used to detect luteinized cells of the corpora lutea; counterstaining was performed with haematoxylin. Note the oocyte trapped in the luteal structure in the cyclin D2<sup>-/-</sup> ovary (arrow). g and h, Dark-field illumination images of e and f, respectively. Abbreviations: g, granulosa cell layer; o, oocyte; l, corpus luteum. Original magnifications, ~140× (a–d) and 100× (e–h).

mutant oocytes resumed meiosis, underwent fertilization following exposure to sperm, cleaved to the two-cell stage, and proceeded to form blastocysts in a pattern that was indistinguishable from that of control oocytes (Table 2).

To determine whether the proliferative failure of granulosa cells in cyclin D2<sup>-/-</sup> ovaries was intrinsic to the follicles, we isolated small follicles of identical size from control and D2<sup>-/-</sup> mice and cultured them *in vitro* in the presence of FSH so that we could study the maturation of ovarian follicles outside the hormonal milieu of the whole animal<sup>8</sup>. The growth *in vitro* of D2<sup>-/-</sup> follicles was greatly impaired compared to that of control follicles (Fig. 3a, b), pointing to a defect intrinsic to the ovarian follicles. This defect could not be attributed to impaired signalling by FSH receptors, because the mutant follicles produced oestradiol at normal levels after FSH treatment (Fig. 3c).

Taken together, these results indicated that cyclin D2 is normally required for the FSH-driven proliferation of granulosa cells. This conclusion was reinforced by studying the expression pattern of the D-type cyclins in the ovaries of normal mice using *in situ* hybridization. We have found that following a single injection of the FSH into these mice, cyclin D2 mRNA, but not D1 or D3 mRNA, was induced in the granulosa cell layer (not shown).

To study more closely how FSH induces cyclin D2 synthesis, we

TABLE 2 *In vitro* culture of oocytes

|                   | Control          | Cyclin D2 <sup>-/-</sup> |
|-------------------|------------------|--------------------------|
| Number of oocytes | 166*<br>(n = 6)† | 118*<br>(n = 5)†         |
| GVB‡ (%)          | 87               | 86                       |
| MII§ (%)          | 44               | 58                       |
| Fertilized   (%)  | 43               | 31                       |
| Blastocysts¶ (%)  | 38               | 55                       |

Germinal vesicle-stage oocytes were isolated from 24-d-old unprimed cyclin D2<sup>-/-</sup> (control) and D2<sup>-/-</sup> mice and subjected to *in vitro* maturation, fertilization and embryo development as described<sup>7</sup>.

\* Total number of oocytes isolated and used for *in vitro* culture.

† Number of females from which oocytes were isolated.

‡ Percentage of cultured oocytes that underwent germinal vesicle breakdown (which is indicative of the resumption of meiosis).

§ Percentage of oocytes undergoing germinal vesicle breakdown that arrested in the metaphase of the second meiotic division.

|| Percentage of oocytes undergoing germinal vesicle breakdown that became fertilized *in vitro* and cleaved to form 2-cell embryos.

¶ Percentage of fertilized oocytes that formed blastocysts.

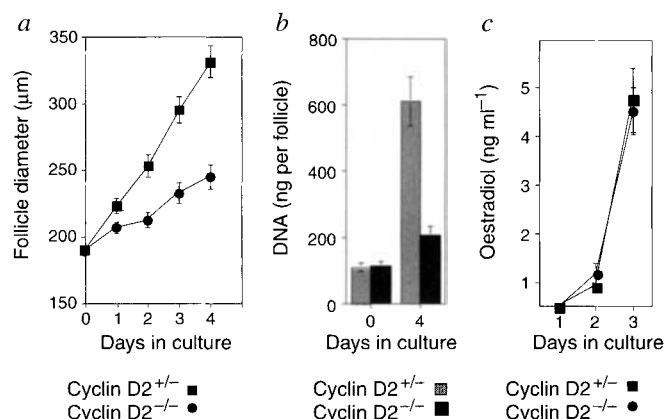


FIG. 3 *In vitro* culture of ovarian follicles. Small preantral follicles were dissected from ovaries of 21-d-old cyclin D2<sup>-/-</sup> (control) and cyclin D2<sup>-/-</sup> mice and cultured for 4 d in the presence of FSH as described<sup>8</sup>. a, Mean follicle diameter; b, mean DNA content per follicle; c, mean concentration of oestradiol-17β in follicle culture medium. Error bars in a–c indicate s.e.

analysed the kinetics of FSH-mediated cyclin D2 mRNA induction by isolating granulosa cells from the ovaries of wild-type rats and culturing them *in vitro*. Rat ovaries were used in this instance because of the greater ease they afford in isolating large numbers of granulosa cells.

As shown in Fig. 4a (lane 1), granulosa cells that were starved by culturing in serum-free medium for 18 h expressed very low levels of cyclin D2 mRNA. Addition of FSH to the culture medium led to a rapid induction of cyclin D2 that was evident within 1 hour of FSH stimulation (Fig. 4a, lane 2) and increased further in the following hour (Fig. 4a, lane 3). No cyclin D1 or D3 transcripts were detected when the same blot was hybridized with appropriate probes (not shown), reinforcing the *in vivo* results which indicated that FSH affects cyclin D2 specifically.

The actions of FSH are mediated by the FSH receptor, a seven-membrane-spanning (serpentine) receptor that is coupled through heterotrimeric G proteins to adenylyl cyclase<sup>6</sup>. Many aspects of FSH receptor activation can be mimicked by treating cells with drugs that raise intracellular cAMP and thereby activate protein kinase A (PKA)<sup>6</sup>. We therefore tested whether cyclin D2 mRNA could be induced by treatment of granulosa cells by forskolin, a direct activator of adenylyl cyclase.

We found that treatment of cultured, mitogen-starved granulosa cells with forskolin caused cyclin D2 mRNA induction (Fig.

4b, lanes 2 and 3), with kinetics and magnitude that paralleled induction by FSH (Fig. 4b, lane 4). In contrast, treatment with the peptide gonadotropin-releasing hormone, which specifically activates the protein kinase C (PKC) pathway in granulosa cells<sup>6</sup>, failed to induce cyclin D2 significantly (Fig. 4b, lane 5). Furthermore serum, which contains many mitogenic growth factors, did not markedly increase cyclin D2 mRNA (Fig. 4a, lane 4). Thus, cyclin D2 mRNA was specifically induced by the PKA but not by the PKC signalling pathway, or by growth factor-associated pathways in granulosa cells. The rapidity of cyclin D2 induction in response to FSH and forskolin suggests that these agents act directly by an intracellular signal transduction cascade on the cyclin D2 gene, rather than indirectly through activation of an autocrine signalling loop.

Although the mitogenic effects of cAMP on some endocrinal cell types have long been known<sup>9</sup>, the mechanism of this mitogenic action has not. Our results explain cAMP effects on cell proliferation by demonstrating that this second messenger can induce expression of a critical component of the cell cycle machinery, cyclin D2. This cAMP-cyclin D2 signalling pathway may also operate in other endocrinal cell types in which cAMP serves as a mitogenic second messenger.

In addition to its role in ovarian follicular development, FSH also functions in male spermatogenesis<sup>10</sup>. We therefore examined the testicular development in D2<sup>-/-</sup> males, although there was no impairment in their fertility. The weight of testes at birth was the same in wild-type and D2<sup>-/-</sup> males, but during sexual maturation the testes of D2<sup>-/-</sup> males grew to a weight that was 2.5 times less than those of wild-type mice, explaining the 2.0–2.9-fold lower sperm count of the mutant males (not shown). Serum testosterone levels did not differ between wild-type and mutant males (not shown), ruling out testosterone deficiency as a cause of testicular hypoplasia. Despite their smaller size, the testes of D2<sup>-/-</sup> mice were normal in their microscopic structure (not shown), indicating that cell differentiation in the mutant testes was normal. Others have recently shown that cyclin D2 expression in the developing testes is found in FSH-responsive cell populations, namely Sertoli cells and spermatogonia<sup>11</sup>. Hence, the second known FSH-responsive organ, the testis, expresses cyclin D2 and clearly depends on this cyclin for normal growth.

In contrast to cyclin D1, no specific involvement of cyclins D2 and D3 in human malignancies has been found. The mammary glands of cyclin D1-deficient mice fail to proliferate normally<sup>3,4</sup>, and cyclin D1 is overexpressed in many human mammary carcinomas<sup>12,13</sup>. Thus, a cyclin that is essential for the normal development of a tissue appears to contribute to neoplastic growth when overexpressed in the same tissue. We therefore asked whether the same functional symmetry might operate in the ovaries and testes.

We determined the amounts of cyclin D2 mRNA in human ovarian tumours of different histological types. Strikingly, ten out of twelve ovarian granulosa cell tumours expressed very high levels of cyclin D2 mRNA compared with normal ovaries (Fig. 5a, b). In contrast, human ovarian cancers of other histological types had very low or undetectable cyclin D2 mRNA levels (except for one tumour, see Fig. 5b). Apart from high cyclin D2 mRNA levels, granulosa cell tumours expressed very little cyclin D1 (Fig. 5a) and D3 (not shown).

We also investigated the expression of cyclin D2 in cell lines derived from human testicular germ-cell (TGC) tumours. Northern blotting analysis revealed that several of these tumour cell lines expressed very high levels of cyclin D2 mRNA; these cell lines expressed virtually no cyclin D1 (Fig. 5c) and very low levels of cyclin D3 (not shown). Strikingly, four out of five cell lines that express high cyclin D2 levels belong to the same histological subtype, being derived from yolk sac tumours. This suggests that cyclin D2 mRNA overexpression may function in the development of a subset of testicular carcinomas.

We also determined the copy number of the cyclin D2 gene in cell lines derived from human testicular germ cell tumours by using Southern blot analysis of their genomic DNA with cyclin D2

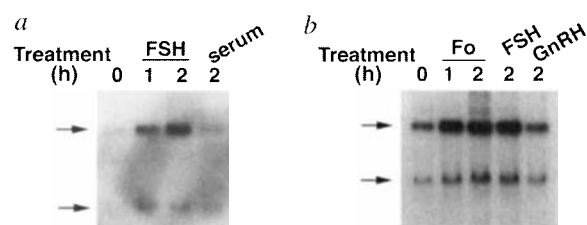


FIG. 4 Induction of cyclin D2 mRNA by FSH and the cAMP-dependent pathway. *a* and *b*, Northern blot analysis of granulosa cells isolated from normal rats and cultured *in vitro*. After starvation in serum-free medium, FSH, 5% fetal bovine serum (serum), forskolin (Fo), or gonadotropin-releasing hormone (GnRH) was added. RNA was isolated at the indicated times. Arrows indicate the cyclin D2 mRNA signal.

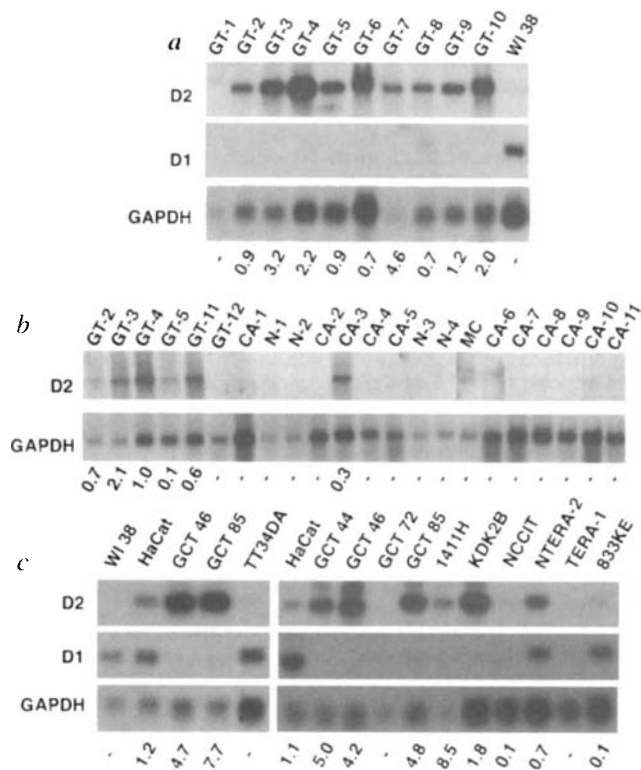


FIG. 5 Cyclin D2 mRNA expression in human ovarian tumours and in testicular germ-cell tumour cell lines. *a*, RNA extracted from human ovarian granulosa cell tumours (GT-1 to GT-10) or from normal human diploid fibroblasts (WI 38) probed with cyclin D2 or cyclin D1 cDNA. GAPDH hybridization was used to normalize the amount of RNA in each lane. *b*, RNA extracted from human ovarian tumours of different histological cell types was probed with cyclin D2 cDNA and GAPDH. GT-1 to GT-12, ovarian granulosa cell tumours; CA-1, CA-4, CA-7 and CA-8, ovarian papillary serous carcinoma; CA-2, CA-3 and CA-11, ovarian clear-cell carcinoma; CA-5 and CA-9, ovarian poorly differentiated carcinoma; CA-6 and CA-10, ovarian endometrioid carcinoma; MC, ovarian mesothelial inclusion cyst; N-1 to N-4, normal human ovary. *c*, Northern blot analysis of RNA extracted from various human testicular germ-cell tumour cell lines, normal diploid fibroblasts (WI 38) and human keratinocytes (HaCat) probed with cyclin D1, D2 and GAPDH. HaCat were included as a positive control as they express very high levels of cyclin D2 mRNA<sup>22</sup>. In *a*–*c*, the signals for cyclin D2 and GAPDH were quantified by densitometry of autoradiographs; the ratio of the band intensities (D2/GAPDH) is shown below each lane (dashes indicate ratios less than 0.05).

complementary DNA as a probe. As an internal standard, we used the cyclin D2 pseudogene, which is located on a different chromosome from the one carrying the active cyclin D2 gene<sup>14</sup>.

An increase in copy number of the cyclin D2 gene (2.4–5.9 fold) was apparent in 15 out of 19 cell lines tested (data not shown). Virtually all TGC tumours are known to have extra copies of the chromosomal region 12p13, which contains the cyclin D2 gene<sup>14</sup>, usually in the form of the isochromosome 12p, i(12p) (refs 15–17). We presume that the extra copies of the cyclin D2 gene that we find in TGC tumour cell lines are contributed by i(12p). However, many TGC tumour cell lines, including some lines with extra copies of chromosome 12p, expressed little or no cyclin D2 mRNA, so it remains to be determined whether cyclin D2 overexpression plays a causal role in some cases of gonadal neoplasia. We suggest, in any event, that agents capable of specifically antagonizing cyclin D2 function might be highly selective in their targeting of testicular tumours and granulosa cell tumours, given the minimal effects of cyclin D2 deletion on all other mouse tissues.

Our results indicate that the expression of a cyclin that is essential for the development of a tissue is particularly susceptible to being subverted during oncogenesis in the same tissue. For this reason, elucidation of the regulatory pathways governing the expression of D-type cyclins is likely to be critical to our understanding of oncogenesis in a variety of tissues. □

## Methods

**Generation of cyclin D2<sup>-/-</sup> mice.** D3 embryonic stem (ES) cells were transfected and selected as described<sup>3</sup>. Fifteen out of 168 ES cell clones analysed underwent homologous recombination at the cyclin D2 locus (Fig. 1b). Three of these D2<sup>-/-</sup> clones contributed to the germline tissue when mutant mice were generated as described<sup>3</sup>. For the genotyping, genomic DNA was digested with NcoI and analysed by Southern blotting using probe A (Fig. 1b,c) or by polymerase chain reaction (PCR) using cyclin D2-specific primers: D (5'-GCT GGC CTC CAA TTC TAA TC-3'), G (5'-CCA GAT TTC AGC TGC TTC TG-3') and a neo gene-specific primer, N2 (5'-CTA GTG AGA CGT GCT ACT TC-3') as described<sup>3</sup> (Fig. 1d). To confirm the absence of cyclin D2 protein in D2<sup>-/-</sup> animals, proteins were extracted from 12.5-days post-coitum mouse embryos, separated using SDS-PAGE, transferred and probed with rat monoclonal anti-cyclin D2 antibody (34B1-3; Santa Cruz Biotechnology), followed by enhanced chemiluminescence detection (Amersham) (Fig. 1e).

**Gonadotropin treatment.** Mice were injected intraperitoneally with FSH administered in the form of 5 IU of pregnant mare serum gonadotropin (Sigma). In some instances, 44–48 h later mice were injected intraperitoneally with LH in the form of 5 IU of human chorionic gonadotropin (Sigma).

**In vitro culture of ovarian follicles.** Total of 26 control and 46 cyclin D2<sup>-/-</sup> follicles were dissected, cultured for 4 d, and the diameters measured daily<sup>8</sup>. Outer follicle diameters (the whole follicle, including the theca layer) are shown in Fig. 3a. Patterns of growth were identical when mean inner diameters (excluding theca cells) were plotted (not shown). For DNA content analysis, a total of eight D2<sup>-/-</sup> follicles and ten control follicles were analysed at day 0; 20 D2<sup>-/-</sup> follicles and 12 control follicles were analysed at day 4. Follicle cells were dissociated with trypsin and pooled (2–3 follicles per assay). DNA content was then determined by fluorometry using Hoechst 33258 (Sigma)<sup>18</sup>. Dilutions of sonicated salmon-testis DNA served to calibrate the assay. Oestradiol levels were measured as described<sup>3</sup> in pools of conditioned medium derived from *in vitro* culture of 8–10 follicles. Day-4 measurements were excluded from the analysis owing to wide variability between pools (for unknown reasons).

**In situ hybridization.** This was done as described<sup>3</sup>. Mouse cyclin D1, D2, D3 and P450sc cDNAs were used to generate <sup>35</sup>S-UTP-labelled riboprobes. The blue colour represents the Hoechst staining of cell nuclei; red represents the cyclin hybridization signal.

**Granulosa cell culture.** Granulosa cells were isolated from Holtzman Sprague Dawley rats and were cultured as described<sup>19</sup> with FSH (100 ng ml<sup>-1</sup>) or forskolin (10 μM) or gonadotropin-releasing hormone (1 μg ml<sup>-1</sup>), or fetal bovine serum (5%). Total RNA was extracted<sup>20</sup>; 20-μg samples were resolved by electrophoresis and transferred. Blots were then stained with acridine orange to confirm equal loading and transfer of RNA samples<sup>19</sup>, and hybridized with radiolabelled mouse cyclin D1, D2 or D3 cDNA.

**Human tumour analysis.** Frozen human tumour samples were obtained from Cooperative Human Tissue Network, Columbus, Ohio. Testicular germ-cell (TGC) tumour cell lines NTERA-2 cl.D1, Tera-2 (both embryonal carcinoma), NCCIT (teratocarcinoma), and normal human diploid fibroblasts WI 38 were purchased from ATCC. Other TGC tumour cell lines were gifts from M. F. Pera: cell lines GCT 27, GCT 35, GCT 48, GCT 63, GCT 67, OX 4 (all embryonal carcinoma) and GCT 44, GCT 46, GCT 72 and GCT 85 (all yolk sac carcinoma);

P. W. Andrews: cell lines 833KE, 1156QE, 2102Ep, Tera-1 (all embryonal carcinoma), 577MF (teratoma) and 1411H (yolk sac carcinoma); J. M. Parrington: cell lines TT34DA and KDK2B (both teratoma); and D. Hogg: cell line 1618K (embryonal carcinoma). Total RNA was isolated from tumours using a single-step guanidinium thiocyanate method<sup>22</sup> or from exponentially growing cells<sup>22</sup>. 10–20 μg of total RNA was resolved by electrophoresis, blotted and probed with human cyclin D1, D2 and D3 cDNA and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as described<sup>22</sup>. DNA was extracted from exponentially growing human TGC tumour cell lines and human diploid fibroblasts (WI 38), digested with BglII restriction endonuclease, resolved by electrophoresis, and blotted and probed with human cyclin D2 cDNA using standard procedures. The ratio of band intensities representing the cyclin D2 gene and the D2 pseudogene was determined for each cell line by densitometry of an autoradiograph and was normalized against the D2 gene/pseudogene ratio in WI 38 normal diploid fibroblasts.

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## Transcription factor GATA-3 is required for development of the T-cell lineage

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**THE zinc-finger transcription factor GATA-3 is expressed in haematopoietic cells and in the developing kidney and nervous system<sup>1–7</sup>. Within the haematopoietic lineages, expression of GATA-3 is restricted to thymocytes and T cells. Functionally important GATA-3 binding sites have been identified in multiple T-cell-specific genes<sup>1,6–8</sup>. Mice containing homozygous null mutations of the GATA-3 gene die on embryonic day 12, precluding a detailed assessment of the role of GATA-3 in haematopoietic development<sup>9</sup>. Here we have used murine embryonic stem (ES) cells containing homozygous mutations in the GATA-3 gene**

(*GATA-3*<sup>-/-</sup>) in conjunction with the *RAG-2*<sup>-/-</sup> (ref. 10) and C57BL/6 complementation systems to study the role of *GATA-3* in mammalian haematopoiesis. Our results show that *GATA-3*<sup>-/-</sup> ES cells can contribute to the development of the mature erythroid, myelomonocytic and B-cell lineages, but fail to give rise to thymocytes or mature peripheral T cells. The differentiation of *GATA-3*<sup>-/-</sup> T cells is blocked at or before the earliest double-negative (CD4<sup>-</sup>/CD8<sup>-</sup>) stage of thymocyte development, such that the *GATA-3*<sup>-/-</sup> ES cells are unable to contribute measurably to the double-negative thymocyte population. These findings suggest that *GATA-3* is an essential and specific regulator of early thymocyte development.

To assess the role of *GATA-3* in murine haematopoiesis, a deletion was introduced into exons 4 and 5 of the *GATA-3* gene in murine ES cells by homologous recombination (Fig. 1a). This deletion resulted in the replacement of the DNA-binding domain of *GATA-3* with a *PGK-neo*<sup>R</sup> cassette. Several independent ES cell clones containing heterozygous deletions of the *GATA-3* gene (*GATA-3*<sup>+/-</sup>) were obtained (Fig. 1b). After selection in high concentrations of G418, we obtained two independent ES cell clones which were shown by Southern blot analysis to contain homozygous deletions of the *GATA-3* gene (*GATA-3*<sup>-/-</sup>) (Fig. 1b). Southern blot analysis with a *neo*<sup>R</sup> probe demonstrated a single integration site for the targeting vector in both clones (data not shown). After 9 days of differentiation *in vitro*, heterozygous *GATA-3*<sup>+/-</sup> ES cells expressed lower levels of mature *GATA-3* messenger RNA than the wild-type (*GATA-3*<sup>+/+</sup>) ES cells (Fig. 1c). More importantly, homozygous *GATA-3*<sup>-/-</sup> ES cells lacked mature *GATA-3* transcripts. Identical results were obtained after 12 and 15 days of *in vitro* differentiation of the *GATA-3*<sup>-/-</sup> ES cells (data not shown), confirming the lack of *GATA-3* expression in these clones.

Each of the *GATA-3*<sup>-/-</sup> ES cell clones was injected into blastocysts with homozygous mutations in the recombinase activating gene-2 (*RAG-2*<sup>-/-</sup>) to produce *GATA-3*<sup>-/-</sup>-*RAG-2*<sup>-/-</sup> chimaeric mice. Because mature B and T lymphocytes cannot develop in the absence of *RAG-2*, this system provides a stringent test of the role of *GATA-3* in murine lymphoid development<sup>10</sup>. In agreement with previous studies<sup>11</sup>, thymi from adult *RAG-2*<sup>-/-</sup> mice contained markedly fewer thymocytes than did normal age-matched control thymi ( $11.8 \times 10^6 \pm 0.7 \times 10^6$ , versus  $194 \times 10^6 \pm 15.7 \times 10^6$  (mean  $\pm$  s.e.m.) for the wild type). By flow cytometry, all of the *RAG-2*<sup>-/-</sup> thymocytes displayed an immature double-negative (CD4<sup>-</sup>/CD8<sup>-</sup>) phenotype (Fig. 2a). Chimaeric mice produced by the injection of *RAG-2*<sup>-/-</sup> blastocysts with heterozygous *GATA-3*<sup>+/-</sup> ES cells (*GATA-3*<sup>+/-</sup>-*RAG-2*<sup>-/-</sup>) displayed increased numbers of thymocytes which, by flow cytometry, were indistinguishable from those of wild-type (*GATA-3*<sup>+/+</sup>) control mice (Fig. 2a); that is, they contained normal proportions of more mature double-positive (CD4<sup>+</sup>/CD8<sup>+</sup>) and single-positive (CD4<sup>+</sup>/CD8<sup>-</sup> and CD4<sup>-</sup>/CD8<sup>+</sup>) cells, as well as normal populations of CD3<sup>+</sup>/TCR $\alpha\beta$ <sup>+</sup> and CD3<sup>+</sup>/TCR $\gamma\delta$ <sup>+</sup> thymocytes. These findings demonstrated that the *GATA-3*<sup>+/-</sup> ES cells could completely rescue the defect in thymocyte development seen in the *RAG-2*<sup>-/-</sup> mice. In contrast, chimaeric animals produced by the injection of *RAG-2*<sup>-/-</sup> blastocysts with *GATA-3*<sup>-/-</sup> ES cells displayed markedly reduced thymocyte numbers that were not significantly different from those of the *RAG-2*<sup>-/-</sup> mice ( $19.8 \times 10^6 \pm 5.7 \times 10^6$  versus  $11.8 \times 10^6 \pm 0.7 \times 10^6$ ) for the *RAG-2*<sup>-/-</sup> mice. Moreover, by flow cytometry, the *GATA-3*<sup>-/-</sup>-*RAG-2*<sup>-/-</sup> thymi, like the *RAG-2*<sup>-/-</sup> thymi, contained only double-negative cells, and lacked more mature double- and single-positive thymocytes as well as CD3<sup>+</sup>, TCR $\alpha\beta$ <sup>+</sup> and TCR $\gamma\delta$ <sup>+</sup> cells (Fig. 2a). The development of *RAG-2*<sup>-/-</sup> double-negative thymocytes is blocked at the CD44<sup>+</sup>/CD25<sup>+</sup> stage<sup>12</sup>. In two-colour flow cytometric analyses with antibodies against CD25 and CD44, the *GATA-3*<sup>-/-</sup>-*RAG-2*<sup>-/-</sup> thymocytes were indistinguishable from the immature *RAG-2*<sup>-/-</sup> thymocytes (data not shown). Finally, and most importantly, Southern blot analyses with a *neo*<sup>R</sup> probe demonstrated that the *GATA-3*<sup>-/-</sup> ES cells contributed to the

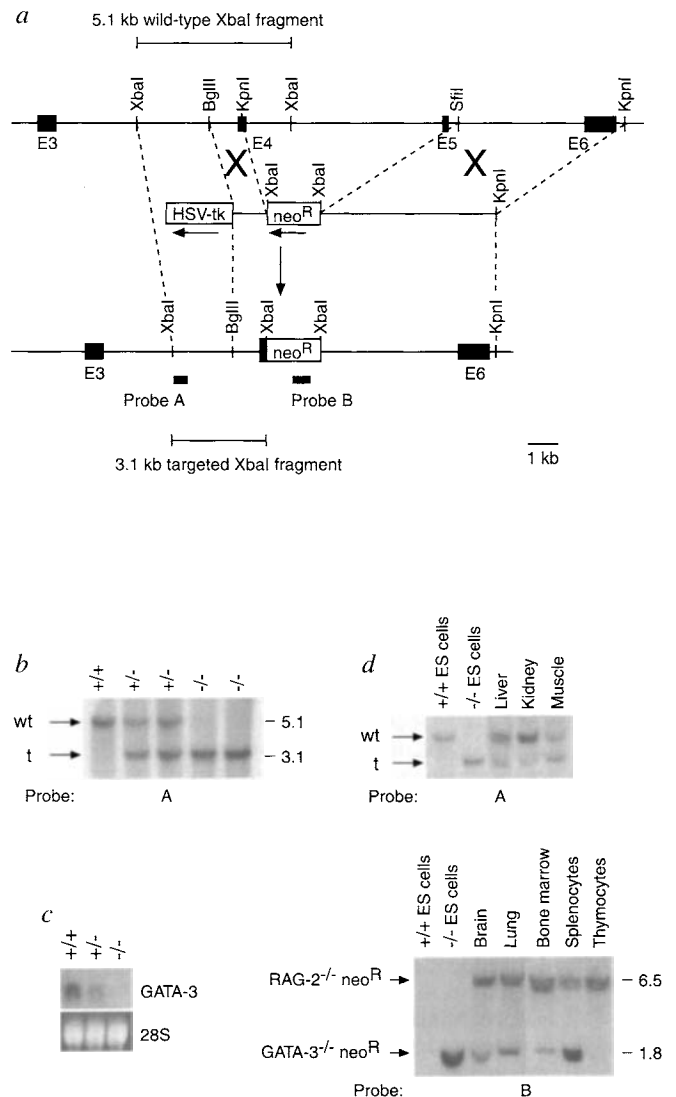


FIG. 1 Targeted disruption of the *GATA-3* gene. *a*, Schematic representation of the *GATA-3* targeting construct. Top, partial restriction endonuclease map of murine *GATA-3*; middle, structure of the *GATA-3* targeting construct containing the HSV-tk and neomycin resistance (*neo*<sup>R</sup>) genes, both under the control of the mouse phosphoglycerate kinase (PGK) promoter; bottom, structure of the targeted *GATA-3* allele which contains a partial deletion of exon 4 and a complete deletion of exon 5. This deletion removes the DNA binding domain of the murine *GATA-3* protein (amino acids 278–349). *b*, Southern blot analysis of the *GATA-3* wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mutant ES cell clones. The 5.1-kb wild-type (wt) and the 3.1-kb targeted (t) alleles are indicated by arrows. *c*, Top, northern blot analysis of *GATA-3* expression in the wild-type, heterozygous and homozygous *GATA-3* mutant ES cell clones after 9 days of differentiation into embryoid bodies *in vitro*. Bottom, equal loading and integrity of the RNA samples was confirmed by ethidium bromide staining of the 28S RNA in the gel before transfer to nitrocellulose membrane (28S). *d*, Southern blot analysis of the contribution of the *GATA-3*<sup>-/-</sup> ES cells to different tissues and to single-cell thymocyte preparations in adult *GATA-3*<sup>-/-</sup>-*RAG-2*<sup>-/-</sup> chimaeric mice. Top, Southern blot was probed with the genomic *GATA-3* probe A (see Fig. 1a), which detects both the wild-type (wt) and targeted (t) *GATA-3* alleles. Bottom, the blot was probed with the *neo*<sup>R</sup> probe (B), which detects a 1.8-kb band derived exclusively from the *GATA-3*<sup>-/-</sup> ES cells and a 6.5-kb band derived from the original *RAG-2* targeting event. The equal intensities of this 6.5-kb band in each sample serve as an internal control for the equal loading of the DNA samples. The 1.8-kb *neo*<sup>R</sup> band is present in brain, lung, bone marrow and splenocytes, but absent from the single-cell thymocyte DNA preparation.



brain, lung, bone marrow and splenocytes, but failed to contribute measurably to the thymocyte-population in the  $GATA-3^{-/-}$ - $RAG-2^{-/-}$  chimaeric animals (compare the intensities of the 1.8-kilobase *neo<sup>R</sup>* bands in Fig. 1d). Similar results were seen in chimaeric mice produced from both of the independently derived  $GATA-3^{-/-}$  ES cell clones. Taken together, these findings suggested that the  $GATA-3^{-/-}$  cells were blocked at the earliest stages of T-cell differentiation, such that they were unable to populate the thymus stably, or to rescue the defect in thymocyte development seen in the  $RAG-2^{-/-}$  animals.

Several experiments were performed to confirm the specificity of the defect in thymocyte development observed in the  $GATA-3^{-/-}$ - $RAG-2^{-/-}$  animals. First, as described above, Southern blot analyses demonstrated that the  $GATA-3^{-/-}$  ES cells contributed significantly to multiple tissues in the chimaeric mice including brain, liver, kidney, lung, bone marrow, splenocytes and skeletal muscle (Fig. 1d). By this analysis, all of the mice used in these experiments were judged to be 10–50% chimaeric. Thus the thymocyte developmental defect observed in the  $GATA-3^{-/-}$ - $RAG-2^{-/-}$  chimaeras did not reflect a generalized defect in the developmental potential of these ES cell clones, nor inadequate chimaerism in these animals. Second, flow cytometric analyses of splenocytes demonstrated normal populations of mature single-positive and  $CD3^{+}/TCR\alpha\beta^{+}$  splenocytes in both wild-type ( $GATA-3^{+/+}$ ) and  $GATA-3^{+/-}$ - $RAG-2^{-/-}$  control mice, but a complete absence of splenic (Fig. 2b) and circulating T cells (data not shown) in the  $GATA-3^{-/-}$ - $RAG-2^{-/-}$  mice. Thus the severe defect in thymocyte development seen in the  $GATA-3^{-/-}$ - $RAG-2^{-/-}$  mice was reflected in the absence of peripheral T cells in these animals. Perhaps most importantly, both the spleen and bone marrow from the  $GATA-3^{-/-}$ - $RAG-2^{-/-}$  mice contained normal populations of  $B220^{+}/IgM^{+}$  mature B cells (Fig. 2b, c). These B cells seemed functionally intact in that they proliferated normally in response to both lipopolysaccharide treatment and IgM crosslinking. Moreover, they generated normal levels of serum IgG and IgA, and increased levels of serum IgM in the  $GATA-3^{-/-}$ - $RAG-2^{-/-}$  chimaeric mice (data not shown). Taken together, these experiments demonstrated that the  $GATA-3^{-/-}$  ES cells were able to rescue the defect in B-cell development seen in the  $RAG-2^{-/-}$  mice, but selectively failed to reconstitute the T-cell populations in these same animals.

Previous studies of mice containing homozygous germline mutations of the *GATA-3* gene suggested that they might display a generalized defect in haematopoiesis<sup>9</sup>. This conclusion was based upon the finding that  $GATA-3^{-/-}$  fetal liver cells from mice at embryonic day 11.5 formed decreased numbers of haematopoietic colonies during culture *in vitro*. However, it was not clear whether this finding reflected a cell-intrinsic defect in the  $GATA-3^{-/-}$  haematopoietic precursors or a more generalized microenvironmental defect in the  $GATA-3^{-/-}$  mice. To address this question directly,  $GATA-3^{-/-}$  ES cells were injected into C57BL/6 blastocysts, and the contributions of the ES cells to different haematopoietic lineages were assayed in the resulting chimaeric animals. Three different assays were used in these experiments. First, because the 129 strain (from which the  $GATA-3^{-/-}$  ES cells were derived) is  $Ly9.1^{+}$ , whereas the C57BL/6 strain is  $Ly9.1^{-}$ , we used two-colour flow cytometry with an anti- $Ly9.1$  antibody and antibodies specific for different haematopoietic lineages to analyse thymocytes, splenocytes and bone-marrow cells from the chimaeric mice (Fig. 3a–c). In accord with our findings in the  $GATA-3^{-/-}$ - $RAG-2^{-/-}$  chimaeras, the  $GATA-3^{-/-}$  ES cells failed to generate double- or single-positive  $Ly9.1^{+}$  T cells (as assayed by

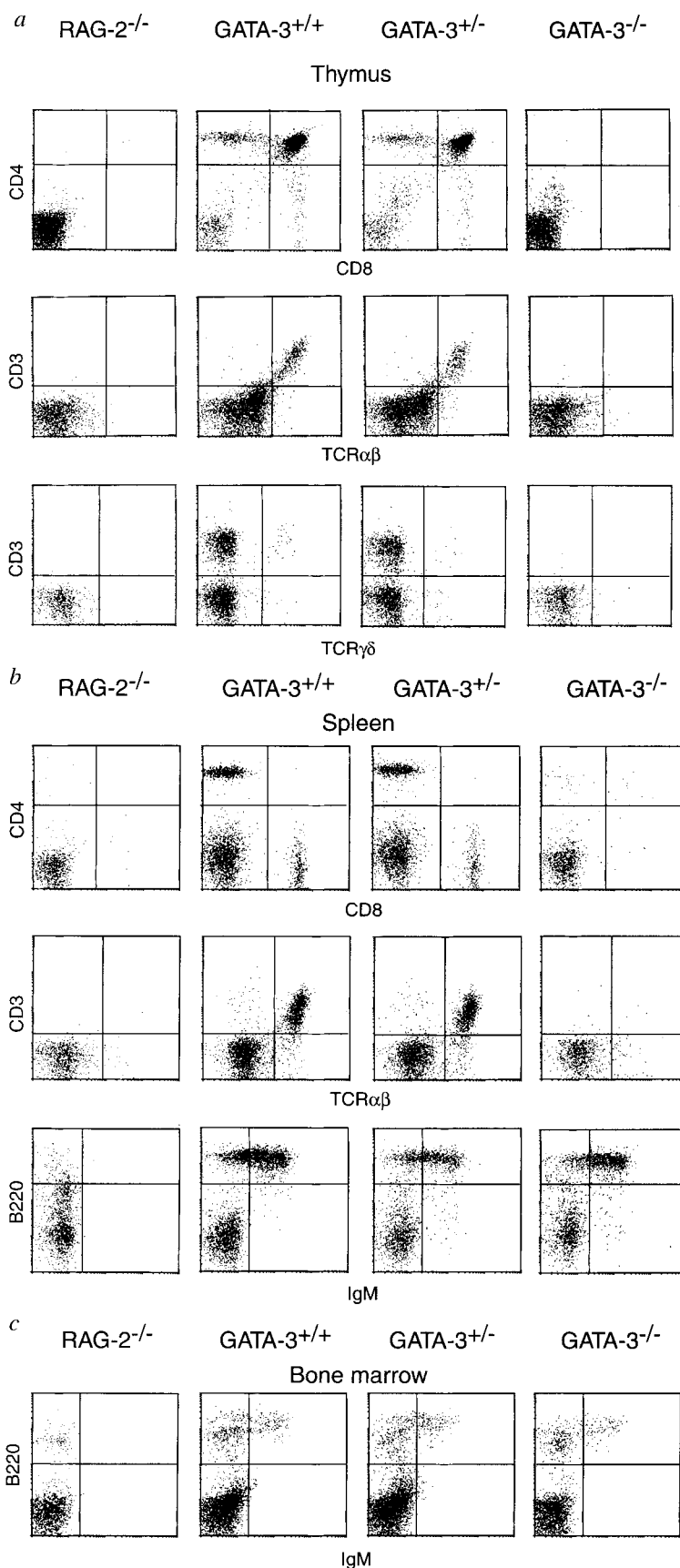


FIG. 2 Flow cytometric analyses of lymphocytes from  $GATA-3^{-/-}$ - $RAG-2^{-/-}$  chimaeric mice. Fluorescence staining profiles of lymphocytes from thymus (a), spleen (b) and bone marrow (c) of  $RAG-2^{-/-}$ ,  $GATA-3^{+/+}$ ,  $GATA-3^{+/-}$ - $RAG-2^{-/-}$  ( $GATA-3^{+/-}$ ), and  $GATA-3^{-/-}$ - $RAG-2^{-/-}$  ( $GATA-3^{-/-}$ ) chimaeric mice. Results are representative of 5  $GATA-3^{+/+}$ , 4  $GATA-3^{+/-}$  and 6  $GATA-3^{-/-}$  mice 6–12 weeks old.

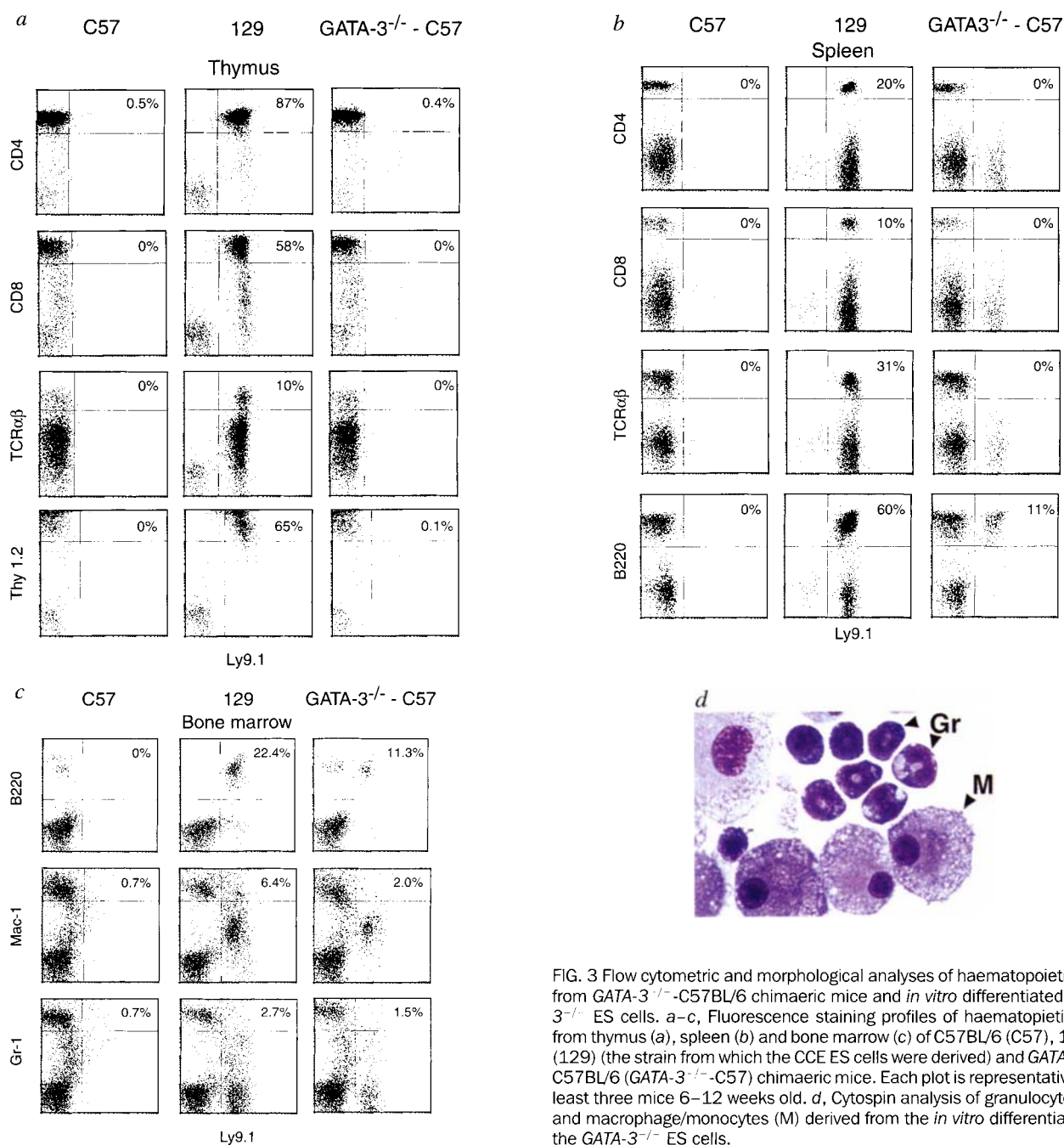


FIG. 3 Flow cytometric and morphological analyses of haematopoietic cells from *GATA-3*<sup>-/-</sup>-C57BL/6 chimaeric mice and *in vitro* differentiated *GATA-3*<sup>-/-</sup> ES cells. *a-c*, Fluorescence staining profiles of haematopoietic cells from thymus (*a*), spleen (*b*) and bone marrow (*c*) of C57BL/6 (C57), 129/Sv (129) (the strain from which the CCE ES cells were derived) and *GATA-3*<sup>-/-</sup>-C57BL/6 (*GATA-3*<sup>-/-</sup>-C57) chimaeric mice. Each plot is representative of at least three mice 6–12 weeks old. *d*, Cytopsin analysis of granulocytes (Gr) and macrophage/monocytes (M) derived from the *in vitro* differentiation of the *GATA-3*<sup>-/-</sup> ES cells.

simultaneous staining with antibodies for CD4, CD8 and TCRαβ) in either the thymi or spleens of the *GATA-3*<sup>-/-</sup>-C57BL/6 chimaeric mice (Fig. 3*a, b*). Furthermore, neither Thy1.2<sup>+</sup>/Ly9.1<sup>+</sup> nor Ly9.1<sup>+</sup> double-negative thymocytes were detectable in these animals, consistent with a block in thymocyte development at the level of the early committed T-cell precursors (Fig. 3*a*, and data not shown). In contrast, the *GATA-3*<sup>-/-</sup> ES cells contributed significantly to the B-cell populations in the spleen and bone marrow (as assessed by staining with an anti-B220 antibody), as well as to the macrophage and granulocyte populations in the bone marrow (as assessed by staining with antibodies for Mac-1 and Gr-1, respectively) (Fig. 3*b, c*) and to the liver, lungs and bone marrow of these mice (as assessed by Southern blot analysis) (Fig. 4*a*). Moreover, after *in vitro* differentiation, the *GATA-3*<sup>-/-</sup> ES cells gave rise to both macrophages and granulocytes as assessed by cytopsin morphology (Fig. 3*d*). The *GATA-3*<sup>-/-</sup> ES cells express the *c* isozyme of the enzyme, glucose phosphate isomerase (GPI-1<sup>c</sup>), whereas C57BL/6 cells express GPI-1<sup>b</sup>. We therefore

used a thin-layer chromatographic assay for GPI to assess the contribution of the *GATA-3*<sup>-/-</sup> ES cells to different tissues and cell lineages in the *GATA-3*<sup>-/-</sup>-C57BL/6 chimaeric mice (Fig. 4*b*). Unlike the Southern blot analysis, which is quantitative, the GPI assay provides only a qualitative assessment of the ES cell contribution. This difference reflects the relative instability of GPI-1<sup>c</sup> as compared to GPI-1<sup>b</sup> (ref. 13). Nevertheless, in keeping with the Southern blot results, the GPI assay demonstrated that the *GATA-3*<sup>-/-</sup> ES cells contributed significantly to brain, lung, liver, splenocytes, bone marrow and the purified red blood cell fraction, but failed to contribute to the thymocyte population in these chimaeric mice.

Previous studies have identified several transcription factors that play important roles in T-cell differentiation<sup>14,15</sup>. One group of factors, including PU.1 (refs 16, 17), Ikaros (ref. 18), AML1 (ref. 19), tal-1/SCL (ref. 20), GATA-2 (ref. 21) and c-myb (ref. 22) are required for the differentiation of T cells as well as multiple other haematopoietic lineages. A second set of transcription

factors, including TCF-1 (ref. 23) and Ets-1 (ref. 24, 25), have been shown to be required for late stages in T-cell development and survival. However, to our knowledge, GATA-3 is the first transcription factor that is required specifically for the earliest stages in thymocyte development, but is not essential for the differentiation of other haematopoietic lineages including erythroid cells, macrophages, granulocytes or B cells. The failure of the *GATA-3*<sup>-/-</sup> ES cells to contribute measurably to the double-negative thymocyte population in the *GATA-3*<sup>-/-</sup>-*RAG-2*<sup>-/-</sup> chimaeras suggests that GATA-3 is required for the survival, proliferation and/or differentiation of early committed T-cell progenitors.

Several T-cell-specific genes are presumptive targets for GATA-3. These include the TCR  $\alpha$ ,  $\beta$  and  $\delta$  genes, as well as the CD8 $\alpha$  gene<sup>1,6-8,26</sup>. TCR $\beta$  gene rearrangement is an early event which occurs in double-negative thymocytes and is required for the further differentiation of these cells into the double-positive thymocyte population<sup>27,28</sup>. Previous studies have suggested that TCR $\beta$  gene rearrangement may be regulated by the transcriptional activation of the unrearranged TCR $\beta$  locus<sup>29</sup>. Thus it is possible that the lack of GATA-3 leads to the failure to activate TCR $\beta$  transcription (and rearrangement), and thereby accounts at least in part for the early developmental defect observed in the *GATA-3*<sup>-/-</sup> thymocytes. GATA-3 might also regulate the expression of other early T-cell genes including TCF-1 and/or RAG-1 and RAG-2 themselves. The identification of these GATA-3 targets represents an important next step in understanding the development pathway regulated by GATA-3. It will also be of interest to understand the molecular pathways that regulate GATA-3 expression during early lymphoid development. In particular, it will be worthwhile to determine the relationships between GATA-3 and early haematopoietic and lymphoid transcription factors such as PU.1, Ikaros, AML1, c-myc, GATA-2 and tal-1. □

## Methods

**Generation and characterization *GATA-3*<sup>-/-</sup> ES cells.** The targeting construct was generated by insertion of a 1.2-kb *Bgl*III-*Kpn*I fragment containing the

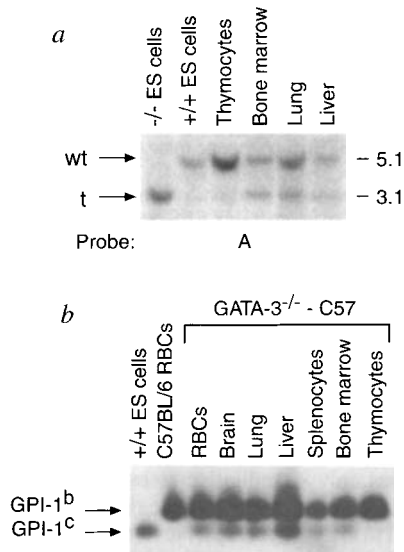
5' end of exon 4 of the GATA-3 gene into the *Kpn*I site of pPNT<sup>30</sup>, followed by ligation of a 5.5-kb *Sfi*I-*Kpn*I fragment containing exon 6 of the GATA-3 gene into the *Not*I/*Xho*I sites of pPNT. The resulting construct was linearized by *Not*I digestion and electroporated into CCE ES cells. Transfectants were selected by growth in G418 (250  $\mu$ g ml<sup>-1</sup>) and gancyclovir (1  $\mu$ M). DNA samples from ES cell clones and tissues from chimaeric mice were characterized by Southern blot analysis using *Xba*I digestion and a radiolabelled 0.4-kb *Hind*III fragment from the GATA-3 gene (probe A, Fig. 1). Homozygous mutant ES cell clones were generated by selection of the heterozygous cells in 1.5–2.0 mg ml<sup>-1</sup> G418. Probes A and B (a 0.4-kb *Pst*I/*Sph*I fragment isolated from pMCleo) were used to determine the contribution of the *GATA-3*<sup>-/-</sup> ES cells to tissues of the chimaeric mice after digestion of tissue DNA samples with *Xba*I. RNA samples were isolated from wild-type and mutant ES cell clones after 9 days of *in vitro* differentiation into embryoid bodies using the TRIzol reagent (GIBCO-BRL) and subjected to northern blot analysis with a radiolabelled GATA-3 cDNA probe (base pairs 1,039–1,244).

**Flow cytometric analyses.** Single-cell suspensions of lymphocytes (0.5  $\times$  10<sup>6</sup>–1.0  $\times$  10<sup>6</sup> cells) were washed twice in PBS with 0.1% NaAzide and incubated in PBS + 0.1% BSA for 20 min on ice with 1  $\mu$ g of anti-Fc receptor (PharMingen) to block Fc $\gamma$ R/III receptors. Cells were then stained for 30 min on ice with phycoerythrin (PE)- and fluorescein isothiocyanate (FITC)-conjugated antibodies. The antibodies used in these experiments included: anti-CD3(500A2), anti-CD4(RM4-5), anti-CD8(53-6.7), anti-Mac-1(M1/70), anti-CD45R/B220(RA3-6B2), anti-Thy1.2(53-2.1), anti-Gr-1(RB6-8C5), anti-Ly9.1(30C7), anti-TCR $\alpha$ (H57-597), anti-TCR $\gamma$ (GL3), anti-IgM(R6-60.2) (PharMingen). Cells were washed once with PBS containing 0.1% NaAzide, and two-colour flow cytometric analyses were performed on a FACScan (Becton-Dickinson). Gating for viable cells was performed using propidium iodide exclusion. Each plot represents analysis of more than 10<sup>4</sup> events using Lysis II software.

***In vitro* differentiation of *GATA-3*<sup>-/-</sup> ES cells.** *GATA-3*<sup>-/-</sup> ES cells were differentiated into haematopoietic colonies by culture in 1% methylcellulose medium supplemented with 10% fetal calf serum, kit ligand (25 ng ml<sup>-1</sup>), erythropoietin (2 U ml<sup>-1</sup>), IL-1 $\alpha$  (750 U ml<sup>-1</sup>), IL-3 (10 ng ml<sup>-1</sup>), GM-CSF (10 ng ml<sup>-1</sup>),  $\alpha$ -monothiolglycerol (0.45 mM), and IL-6 (50 ng ml<sup>-1</sup>) for 20 days with disaggregation and replating after 10 days as described previously<sup>17</sup>. Colonies were collected by centrifugation onto slides with a Cytopro cytocentrifuge (Wescor) at 800 r.p.m. for 5 min and stained with Diff-Quik (Baxter) according to the manufacturer's recommendations. Photomicrographs were obtained with a Nikon microscope (magnification,  $\times$ 1,000).

**GPI assays.** Tissue extracts were assayed for GPI-1 isozymes using cellulose acetate chromatography<sup>13</sup>. Red blood cell fractions were collected following centrifugation of whole blood in microhaematocrit capillary tubes.

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**FIG. 4** Contribution of *GATA-3*<sup>-/-</sup> ES cells to different tissues of the *GATA-3*<sup>-/-</sup>-C57BL/6 chimaeric mice. **a**, Southern blot analysis of *Xba*I-digested DNA extracted from tissues of adult *GATA-3*<sup>-/-</sup>-C57BL/6 chimaeric mice using probe A (see Fig. 1). The 5.1-kb wild-type (wt) and the 3.1-kb targeted (t) alleles are indicated by arrows. **b**, The relative proportions of glucose phosphate isomerase (GPI) isozymes b and c were determined in tissue extracts prepared from adult *GATA-3*<sup>-/-</sup>-C57BL/6 chimaeric mice. Data shown are representative of organs from at least 3 mice. GPI-1<sup>c</sup>, which is derived from the *GATA-3*<sup>-/-</sup> ES cells, is present in red blood cells, brain, lung, liver, splenocytes and bone marrow, but absent from single-cell thymocyte preparations.

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# Nucleotide exchange on ARF mediated by yeast Gea1 protein

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THE ADP-ribosylation factor ARF is a small GTP-binding protein that is involved in the transport of vesicles between the endoplasmic reticulum (ER) and Golgi complex and within the Golgi complex itself<sup>1-4</sup>. ARF cycles between inactive and membrane-associated active forms as a result of exchange of bound GDP for GTP; the GTP-bound form is an essential participant in the formation of transport vesicles<sup>4</sup>. This nucleotide exchange is inhibited by the fungal metabolite brefeldin A (BFA)<sup>5,6</sup>. Here we identify a protein (Gea1) from *Saccharomyces cerevisiae* that is a component of a complex possessing guanine-nucleotide-exchange activity for ARF. We show that the activity of the complex is sensitive to brefeldin A and that Gea1 function is necessary for ER–Golgi transport *in vivo*. Gea1 contains a domain that is similar to a domain of Sec7, a protein necessary for intra-Golgi transport. We propose that Gea1 and ARNO, a human protein with a homologous Sec7 domain<sup>7</sup>, are members of a new family of ARF guanine-nucleotide exchange factors.

The *S. cerevisiae* ARF genes are 72–76% identical to higher eukaryotic ARF proteins<sup>8</sup>, and their function and interactions are probably highly conserved. We constructed two mutants of the *ARF2* gene: G29A and T31N, in which glycine is replaced by alanine at position 29, and threonine by asparagine at position 31, respectively. *ARF2*<sup>G29A</sup> and *ARF2*<sup>T31N</sup> are predicted to bind GTP poorly and to have an increased affinity for their putative guanine-nucleotide-exchange factor. The analogous mutant *Ras2*<sup>G22A</sup> of *S. cerevisiae* has a dominant-negative effect on growth and has a

higher affinity for the Ras exchange factor Cdc25 than for wild-type Ras<sup>9</sup>. Overexpression of Cdc25 suppresses the dominant-negative growth defect caused by either *Ras1*<sup>G22A</sup> or *Ras2*<sup>G22A</sup> (refs. 10, 11). Expression of *ARF1*<sup>T31N</sup> in mammalian cells confers a dominant-negative growth defect on cells, which are indistinguishable from those treated with brefeldin A (BFA)<sup>12</sup>. We found that expression of *ARF2*<sup>G29A</sup> and *ARF2*<sup>T31N</sup> in yeast confers a cold-sensitive, dominant-negative effect on growth (Fig. 1). We identified a plasmid that in multiple copies suppresses the growth defects conferred by *ARF2*<sup>G29A</sup> and *ARF2*<sup>T31N</sup> at the non-permissive temperature (Fig. 1). Subcloning and complementation showed that the gene responsible for this suppression corresponds to *YJR031C* (open reading frame J1580) on chromosome X (ref. 13, and data not shown). A second similar gene (*YEL022*) is found on chromosome V in the *S. cerevisiae* genome. We refer to *YJR031C* as *GEA1* and *YEL022* as *GEA2*, for guanine-nucleotide exchange on ARF. The predicted protein products Gea1 and Gea2 are 1,408 and 1,459 amino acids long, respectively, and are 50% identical. Each has a centrally located region of ~300 amino acids (from residues 500 to 800 of each protein) that is homologous to a domain of the secretion protein Sec7 of *S. cerevisiae*. Sec7 is involved in ER–Golgi and intra-Golgi transport and has been localized to the Golgi apparatus and to ER–Golgi transport vesicles<sup>14,15</sup>. Both human and yeast *ARF* genes suppress the growth and secretion defects of *sec7* temperature-sensitive (*ts*) mutants<sup>16</sup>.

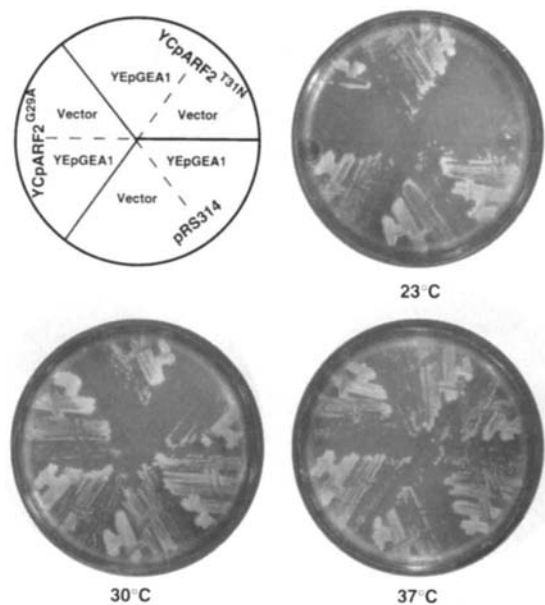


FIG. 1 Cold-sensitive dominant-negative growth phenotypes conferred by *ARF2*<sup>T31N</sup> and *ARF2*<sup>G29A</sup>, and suppression by multicopy *GEA1*. Strain CJY045-5-3 *arf1::HIS3 ARF2* was co-transformed with pCLJ8 ('YEpGEA1') or the parent vector pFL44L ('Vector') and YCpARF2<sup>T31N</sup>, YCpARF2<sup>G29A</sup> or the corresponding PRS314 centromeric vector. Transformants were plated directly onto glucose-containing selective plates and incubated at 37 °C, then shifted to 23 °C.

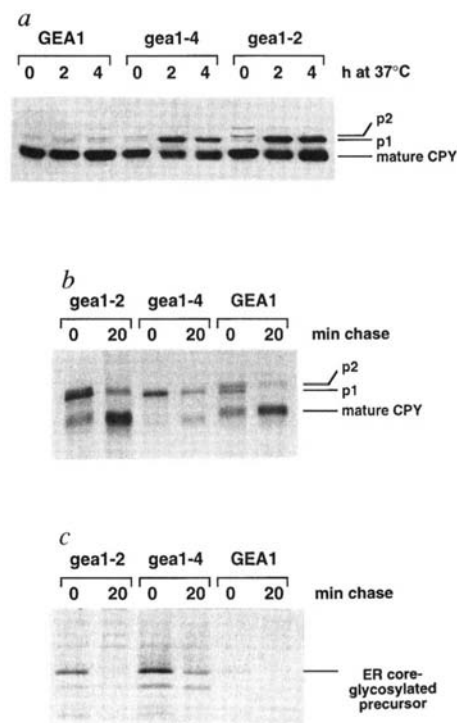


FIG. 2 ER–Golgi transport defect of *gea1* *ts* mutants. **a**, Strains CJY052-10-2 *gea1::HIS3 gea2::HIS3* carrying either the wild-type *GEA1* gene on a multicopy plasmid (pCLJ80) or the *ts* mutant *gea1-2*, *gea1-3* or *gea1-4* (derived from pCLJ80; see Methods) were grown at the permissive temperature (27 °C), then shifted to the restrictive temperature of 37 °C. After 0, 2 or 4 h at 37 °C, aliquots of cells were removed and prepared for western blotting using anti-CPY primary antibodies. Pro-CPY is core-glycosylated in the ER to produce the p1 precursor form, mannosylated in the Golgi apparatus to produce the higher-molecular-weight p2 form, and the pro-region cleaved in the vacuole to produce the lower-molecular-weight mature form<sup>18,27</sup>. **b**, **c**, *GEA1*, *gea1-2* and *gea1-4* carrying cells were incubated for 30 min at 37 °C, then labelled with a mixture of [<sup>35</sup>S]-methionine/cysteine for 10 min, after which an aliquot was removed (0 min chase), followed by addition of cold methionine and cysteine and incubation for an additional 20 min (20 min chase). Cell lysates were prepared from each sample and immunoprecipitated with anti-CPY antibodies (**b**) or with anti- $\alpha$ -factor antibodies (**c**).



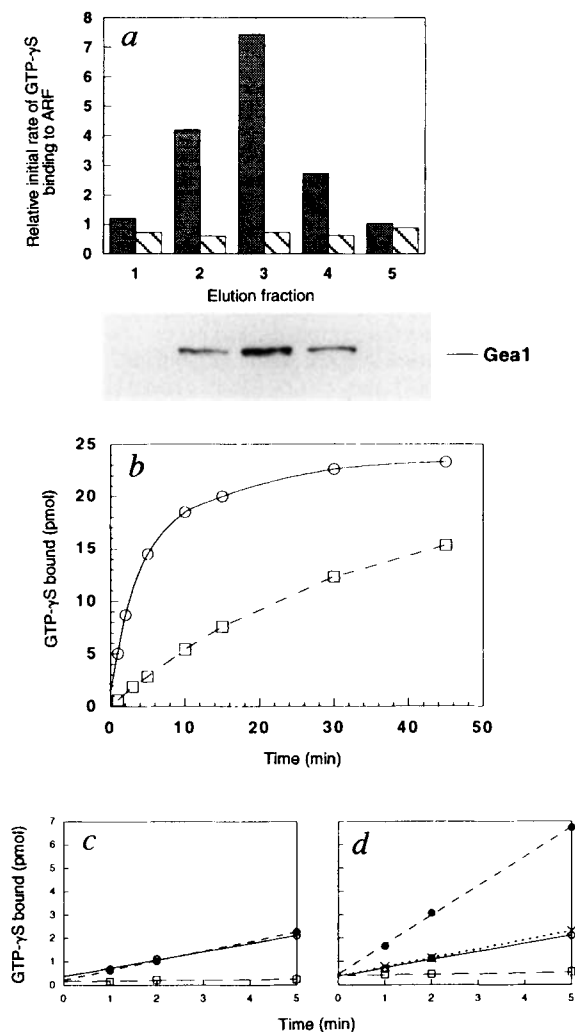


FIG. 3 *a*, Binding of [ $^{35}$ S]GTP $\gamma$ S to 1  $\mu$ M myr-ARF1 in the presence of Ni-NTA column elution fractions from strain CJY52-10-2 *gea1::HIS3* *gea2::HIS3*/pCLJ202 *GAL1::His6-GEA1* (black bars) or from strain CJY50-1 *gea1::HIS3* *GEA2* (hatched bars). The initial velocity of [ $^{35}$ S]GTP $\gamma$ S binding to myr-ARF1 was calculated over the linear range of a time course (*b*, *c*) of [ $^{35}$ S]GTP $\gamma$ S binding in the presence of each elution fraction, and divided by the initial velocity of [ $^{35}$ S]GTP $\gamma$ S binding to myr-ARF1 in the absence of added fraction. Lower panel, western blot showing the amount of Gea1 present in each elution fraction from strain CJY52-10-2/pCLJ202, detected using polyclonal rabbit anti-Gea1 antibodies. *b*, 1  $\mu$ M myr-ARF1 was incubated with [ $^{35}$ S]GTP $\gamma$ S in the presence (circles) or absence (squares) of elution fraction 3 (see *a*) and the amount [ $^{35}$ S]GTP $\gamma$ S bound to myr-ARF1 was determined. 25- $\mu$ l aliquots containing 25 pmol each of myr-ARF1 were analysed for each time point. *c*, *d*, Binding of [ $^{35}$ S]GTP $\gamma$ S to 1  $\mu$ M myr-ARF1 in the presence of Ni-NTA column elution fractions from strain CJY50-1 (*c*) or from strain CJY52-10-2/pCLJ202 (*d*). Incubations are shown with myr-ARF1 alone (open circles), fraction alone (open squares), myr-ARF1 plus fraction (filled circles), and for *d*, myr-ARF1 plus fraction plus 300  $\mu$ M BFA (crosses).

The Sec7 domain is also present in predicted gene products of human and *Caenorhabditis elegans* complementary DNAs, and the *Arabidopsis* *EMB30* gene<sup>13</sup>; *EMB30* has been implicated in cell expansion and cell polarity<sup>17</sup>.

Neither *GEA1* nor *GEA2* is necessary for viability of yeast cells, but deletion of both genes is lethal (data not shown). Temperature-sensitive alleles of *GEA1* were constructed and tested for a secretion defect using carboxypeptidase Y (CPY), a soluble vacuolar enzyme<sup>18</sup>. The wild-type strain accumulated primarily the mature, vacuolar form of CPY and only a small fraction of the precursor p1 (ER) and p2 (Golgi) forms (Fig. 2a). In contrast, the *ts* mutants accumulated significant levels of the p1 but not the p2

precursor after incubation at 37 °C (Fig. 2a). To determine how rapidly transport was blocked, we did pulse-chase analysis after a brief incubation at 37 °C, examining the fates of both CPY and the secreted protein  $\alpha$ -factor<sup>19</sup>. Immediately after the 10-min labelling period, there was a much higher level of both CPY and  $\alpha$ -factor ER precursors in the *gea1 ts* mutants than in the wild-type *GEA1* strain (Fig. 2b, c), indicating that there was a defect in ER–Golgi transport.

We next tested genetic interactions between *GEA1* and other genes involved in this step of transport. We found that multicopy *ARF2* and *ARF1* and, to a lesser extent, *SEC21* partially suppress the *ts* growth defect of *gea1-4*, but that multicopy *SEC7*, *SEC12* or *SEC23* do not suppress *gea1-4* at non-permissive temperatures (data not shown). *SEC21* encodes the  $\gamma$ -subunit of yeast COPII<sup>20</sup>, and *SEC12* and *SEC23* are a GDP/GTP-exchange factor and a GTPase-activating protein, respectively, for the small GTP-binding protein Sar1 which controls COPII binding to ER membranes<sup>3</sup>. These results are consistent with Gea1 having a specific role in COPI function. No suppression was seen by *SEC7*, despite the significant similarity between Sec7 and Gea1. Also, multicopy *GEA1* is unable to suppress the *ts* defect of a *sec7* mutant (data not shown). Hence Sec7 and Gea1 have non-overlapping functions, despite sharing a similar domain.

To purify Gea1, *GEA1* was placed under the control of the strong *GAL1* promoter, and a sequence of six histidine residues (his-tag) was added to the amino terminus of the protein. The his-tagged protein is fully functional *in vivo*, as it complemented the double deletion strain *gea1::HIS3* *gea2::HIS3*. Extracts of cells carrying his-tagged Gea1 and a control extract from a *gea1::HIS3* *GEA2* strain were prepared and passed over Ni-nitrilo-triacetic acid (Ni-NTA) columns in parallel. Elution fractions were then analysed by western blotting to determine the quantity of Gea1 protein present in each fraction, and tested for their capacity to accelerate GTP- $\gamma$ S binding to recombinant bovine myristoylated ARF1 (myr-ARF1) *in vitro*. For the strain expressing his-tagged

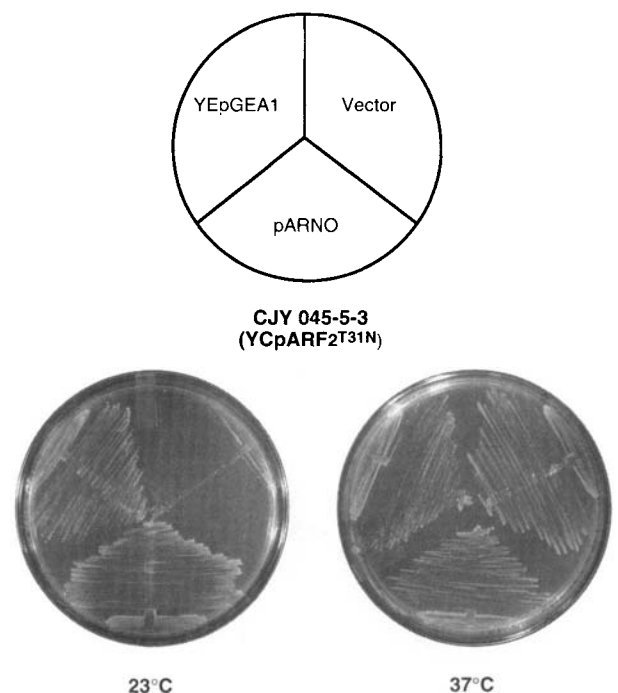


FIG. 4 Expression of human ARNO protein suppresses the dominant-negative growth defect conferred by *ARF2*<sup>T31N</sup> in yeast. Strain CJY045-5-3 was co-transformed with YCpARF2<sup>T31N</sup> and either YE pGEA1, pARNO or the vector 2 $\mu$ p426Met25 (ref. 28). pARNO carries the *ARNO* gene in 2 $\mu$ p426Met25. Transformants were grown at the permissive temperature of 37 °C, then shifted to either 37 °C or the non-permissive temperature of 23 °C on medium lacking methionine to induce expression of ARNO.

Gea1, there was a good correlation between the quantity of Gea1 in each elution fraction and its capacity to stimulate GTP- $\gamma$ S binding to myr-ARF1 (Fig. 3a). The elution fractions from the control strain not expressing his-tagged Gea1 could not accelerate GTP- $\gamma$ S binding to myr-ARF1 with respect to the spontaneous exchange rate (Fig. 3a). The active fractions from the his-tagged Gea1 column contained numerous proteins, as assessed by silver staining. However, the contaminating proteins were identical to those from the control column and thus do not contribute to ARF exchange activity. The time course of GTP- $\gamma$ S binding to myr-ARF1 in the presence and absence of elution fraction 3 from the his-tagged Gea1-containing extract is shown (Fig. 3b). This activity is completely abolished by BFA (Fig. 3c, d), with half-maximal inhibition at 10  $\mu$ M BFA and full inhibition requiring 300  $\mu$ M BFA. Similar results have been obtained for mammalian ARF exchange-factor activities<sup>5,6,21,22</sup>. We determined by gel filtration analysis that Gea1 present in the Ni-NTA column elution fractions is not in a monomeric form, but rather is part of a complex of *M*<sub>r</sub> 500K–600K (data not shown).

In an accompanying paper<sup>7</sup>, the purification of a human protein (ARNO) possessing ARF guanine-nucleotide-exchange activity is reported. Expression of ARNO in yeast suppresses the dominant-negative effect of ARF2<sup>T31N</sup> on growth (Fig. 4). However, ARNO cannot replace Gea1/2 function, as its expression in yeast fails to complement the *ts* growth defect of *gea1-4* and to suppress deletion of both *GEA* genes (data not shown). The only sequence in common between Gea1 and ARNO is the Sec7 domain, implying that this domain may be responsible for ARF exchange activity. Indeed, the Sec7 domain of ARNO is sufficient for ARF1 GDP/GTP-exchange activity *in vitro*<sup>7</sup>. □

## Methods

**ARF2<sup>T31N</sup> suppressor selection.** ARF2<sup>G29A</sup> and ARF2<sup>T31N</sup> genes present on centromeric vectors (YCpARF2<sup>G29A</sup> and YCpARF2<sup>T31N</sup>) were transferred into strain CJY045-8-4 which carries a chromosomal deletion of ARF1, and a plasmid-borne copy of ARF1 under control of the inducible GAL1 promoter. When grown in galactose, the latter is highly expressed and hence the mutant ARF2 protein represents only a small percentage of total ARF protein in the cell. Upon shift to glucose, expression of ARF1 is stopped and the wild-type and mutant ARF2 proteins are then expressed equally. Strain CJY045-8-4 *arf1::HIS3/pRB1302GAL1::ARF1*-YCpARF2<sup>T31N</sup> was transformed with a yeast genomic library carried in the 2-micron vector pFL44L (gift from F. Lacroute)<sup>23</sup>. Of ~17,000 total transformants, 45 colonies were obtained on glucose-containing selective plates at 23 °C. Plasmids from 35 of these colonies were isolated, transferred to *E. coli* and analysed. 24 plasmids carried either the ARF1 or ARF2 gene, 5 carried the TRP1 gene (the YCpARF2<sup>T31N</sup> plasmid carries TRP1 as its selective marker), 4 carried SCM2 encoding tryptophan permease, and one (pCLJ8) carried *GEA1*. We verified that suppression by pCLJ8 was not due to a complete bypass of the requirement for ARF1 and ARF2 in the cell, and that pCLJ8 is able to suppress the dominant-negative growth defect of ARF2<sup>T31N</sup> only in the presence of wild-type ARF1 or ARF2 protein in the cell (data not shown). Yeast genetic methods have been described<sup>24</sup>.

**Construction of *ts* mutants.** *gea1* alleles were constructed in pCLJ80 (2  $\mu$  LEU2 *GEA1*) by a mutagenic PCR plasmid-shuffling protocol<sup>25</sup> as described<sup>26</sup>.

**Western blotting and immunoprecipitation.** For western analysis, cells were lysed by agitation in the presence of glass beads, and lysates loaded directly onto 8% (CPY and Gea1) or 12% ( $\alpha$ -factor) SDS-polyacrylamide gels. After transfer to nitrocellulose membranes, bands were revealed using ECL chemiluminescence (Amersham) according to the supplier's instructions. Radiolabelling and immunoprecipitation were done as before<sup>24</sup>, except that <sup>35</sup>S-labelled amino acids were purchased from Amersham.

**Gea1 purification.** Strains CJY50-1 and CJY52-10-2/pCLJ202 (3-litre cultures of each) were grown to an absorbance at 600 nm of 0.8, the cells were collected and resuspended in 10 ml final volume of buffer A (100 mM NaCl, 20 mM HEPES, pH 7.5, 10% glycerol) containing 2  $\mu$ g ml<sup>-1</sup> leupeptin, aprotinin, chymostatin and pepstatin and 1 mM PMSF, and lysed using a French press. Lysates were cleared of cellular debris, spun at 100,000g and the supernatants recovered. Ammonium sulphate was added to 50% saturation, the pellet was resuspended in buffer A containing protease inhibitors, and then passed over a 200–400  $\mu$ l Ni<sup>2+</sup>-NTA column (Qiagen) prepared according to the supplier's instructions. After washing the column with 10–20 column-volumes of buffer A, followed by 20 column-volumes of buffer A plus 20 mM imidazole, the bound proteins were eluted by passing five 1-column-volume aliquots of buffer A plus 100 mM imidazole over the column.

**GDP-GTP- $\gamma$ S exchange assays.** [<sup>35</sup>S]GTP- $\gamma$ S binding assays were performed using recombinant myristoylated bovine ARF1 in the presence of azolectin vesicles (1.5 g l<sup>-1</sup>) and 1 mM MgCl<sub>2</sub>, as described<sup>22</sup>. The concentration of myr-ARF1 in each reaction was 1  $\mu$ M; samples of 25  $\mu$ l (25 pmol myr-ARF1) were removed at the indicated times.

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## A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains

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**THE small G protein ARF1 is involved in the coating of vesicles that bud from the Golgi compartments<sup>1,2</sup>. Its activation is controlled by as-yet unidentified guanine-nucleotide exchange factors<sup>3,4</sup>. Gea1, the first ARF exchange factor to be discovered in yeast<sup>5</sup>, is a large protein containing a domain of homology with Sec7, another yeast protein that is also involved in secretion<sup>6</sup>. Here we characterized a smaller human protein (relative molecular mass 47K) named ARNO, which contains a central Sec7 domain that promotes guanine-nucleotide exchange on ARF1. ARNO also contains an amino-terminal coiled-coil motif and a carboxy-terminal pleckstrin-homology (PH) domain. The PH domain mediates an enhancement of ARNO exchange activity by negatively charged phospholipid vesicles supplemented with phosphatidylinositol biphosphate. The exchange activity of ARNO is not inhibited by brefeldin A, an agent known to block vesicular transport and inhibit the exchange activity on ARF1 in cell extracts<sup>7,8</sup>. This suggests that a regulatory component which**



FIG. 1 Domain structure of the ARNO protein. *a*, Amino-acid sequence. The coiled-coil (dotted line), Sec7 (double line) and PH (single line) domains are underlined (accession number, X99753). *b*, The Sec7-homology domain present in human ARNO, *Arabidopsis* Emb30 (ref. 10), a *C. elegans* protein<sup>11</sup> deduced from the sequence of cosmid K06H7, the

yeast Sec7 (ref. 6) and Gea1 proteins (ref. 5). Identical amino acids are boxed. A strictly conserved residue mutated in a deficient Emb30 allele<sup>10</sup> is indicated by an asterisk. Note the small number of insertions and deletions, except for a large insert present in Gea1.

is sensitive to brefeldin A associates with ARNO *in vivo*, possibly through the amino-terminal coiled-coil. We propose that other proteins with a Sec7 domain regulate different members of the ARF family.

We found partial sequences for a human homologue of the yeast ARF exchange factor Gea1 (ref. 5) in the expressed sequence tag (EST) database from the IMAGE consortium<sup>9</sup> and determined its complete sequence (1.5 kilobases of complementary DNA). The encoded protein (ARNO, for ARF nucleotide-binding-site opener) is 399 amino acids long, with a predicted  $M_r$  of 47K. It contains a coiled-coil-forming sequence, followed by a domain homologous to Sec7 and Gea1, and a C-terminal PH domain (Fig. 1*a*). The central Sec7 domain of ARNO shares homology with Emb30 (a plant protein<sup>10</sup>), a *Caenorhabditis elegans* protein<sup>11</sup>, yeast Sec7 and Gea1 (Fig. 1*b*). This Sec7 domain defines a new protein motif present in most eukaryotes, including yeast, plants, worms and mammals. We expressed ARNO in *Escherichia coli* and found a high exchange activity for ARF1 in the crude soluble extract. The purified protein (Fig. 2*a*) has  $M_r$  47K by SDS-PAGE, as expected, but runs as a single sharp peak of apparent  $M_r$  90K on gel filtration, indicating that it forms a dimer. ARNO catalyses the rapid exchange of GDP for GTP- $\gamma$ S on myristoylated ARF1 in the presence of azolectin vesicles (Fig. 2*b*), but not on non-myristoylated ARF, which is consistent with our discovery that the exchange activity in crude cell extracts requires myristoylation of ARF and phospholipids<sup>3</sup>. ARNO is inactive on the ARF-related protein ARL3 (Fig. 2*c*). The fungal metabolite brefeldin A (BFA) inhibits ARF exchange in crude cell extracts<sup>3,7,8</sup>, but not the exchange activity of ARNO (Fig. 2*b*), consistent with another observation that sensitivity to BFA is lost in the last purification steps of the exchange activity present in a bovine brain extract<sup>4</sup>. Like ARF1, ARNO is ubiquitously expressed (data not shown). Another protein of very similar sequence (67% identity) was found by searching the data banks<sup>12</sup>; we expressed this protein in bacteria and found a BFA-insensitive exchange activity for ARF1,

comparable to that of ARNO. But as this protein is mostly expressed in haematopoietic cells<sup>12</sup>, ARNO is probably the principal exchange factor for ARF1 in most cell types and may associate with a regulatory protein that would be the BFA-sensitive component.

In the PH domain, the highest homology is found with pleckstrin, followed by other PH domains from dynamin, phospholi-

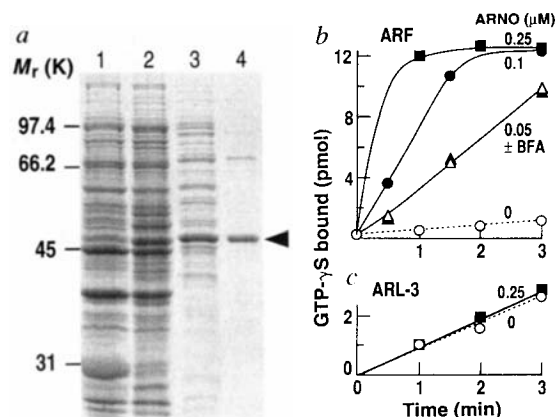
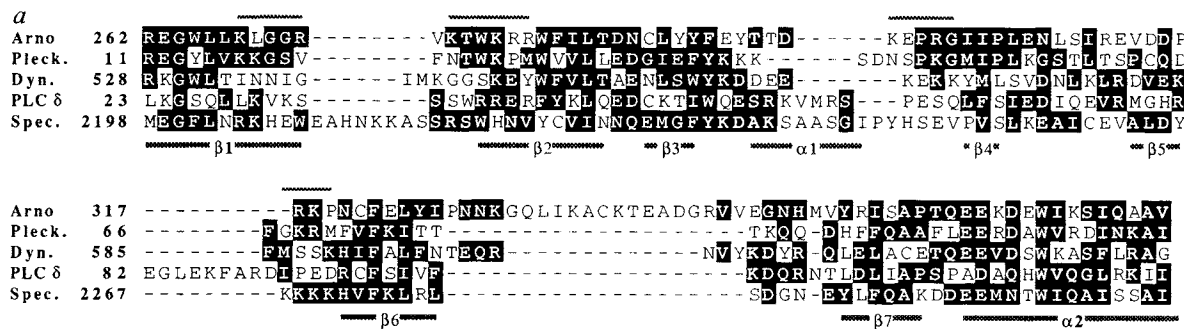
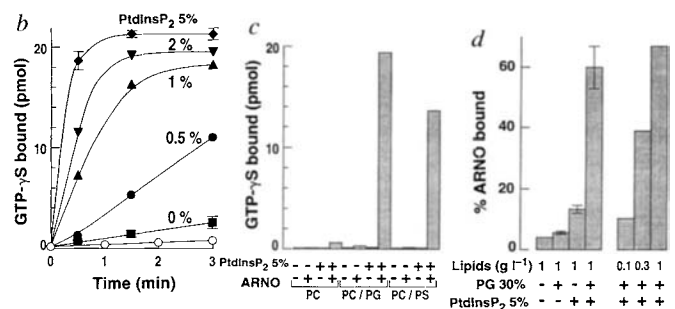


FIG. 2 Stimulation of nucleotide exchange on ARF1 by ARNO. *a*, Purification of ARNO expressed in *E. coli*. Lanes: 1, uninduced, and 2, induced *E. coli* extracts; 3, fractions pooled from the QAE column; and 4, purified ARNO after the gel-filtration column. *b*, Stimulation of GDP to GTP- $\gamma$ S exchange on ARF (0.6  $\mu$ M) by increasing concentrations of ARNO:  $\circ$ , buffer alone;  $\Delta$ , 0.05  $\mu$ M;  $\bullet$ , 0.1  $\mu$ M;  $\blacksquare$ , 0.25  $\mu$ M. The lack of effect of 300  $\mu$ M BFA ( $\Delta$ ) on stimulation by 0.05  $\mu$ M ARNO is also shown. Each point corresponds to a 25- $\mu$ l sample containing 15 pmol ARF1. *c*, GDP to GTP- $\gamma$ S exchange on the ARF-related protein ARL3 (1  $\mu$ M), in the absence ( $\circ$ ) or presence ( $\blacksquare$ ) of 0.25  $\mu$ M ARNO.



**FIG. 3** Effect of PtdInsP<sub>2</sub> on exchange activity and membrane binding of ARNO. **a**, The pleckstrin-homology domain of ARNO compared with those of pleckstrin, dynamin, phospholipase C $\delta$  and spectrin<sup>13,14</sup>. Homologous amino acids are boxed. The large insert present only in ARNO corresponds to loop  $\beta$ 6/ $\beta$ 7 in the structure of PH domains<sup>13,14</sup>. Positively charged regions that could be involved in PtdInsP<sub>2</sub> binding are indicated by thin lines over the sequence. **b**, Stimulation of GDP to GTP- $\gamma$ S exchange on ARF (1  $\mu$ M) by ARNO (75 nM) in the presence of phosphatidyl choline (PC) vesicles containing 30% phosphatidyl glycerol (PG) and increasing concentrations of phosphatidyl inositol-4,5-bis phosphate (PtdInsP<sub>2</sub>): ■, no PtdInsP<sub>2</sub>; ●, 0.5%; ▲, 1%; ▼, 2%; ◆, 5%. Total lipid concentration was 1 mg ml<sup>-1</sup>. The rate of spontaneous exchange in the absence of ARNO (○) was unaffected by PtdInsP<sub>2</sub>. Each point corresponds to a 25- $\mu$ l sample containing 25 pmol ARF1. **c**, Exchange activity on ARF measured as the amount of GTP- $\gamma$ S bound in 30 s with 75 nM ARNO, in the presence of vesicles (1 mg ml<sup>-1</sup> lipid) containing PC, PC + 30% PG, or PC + phosphatidyl serine (PC + 30% PS), with (+) or without (-) 5% PtdInsP<sub>2</sub>. **d**, Sedimentation of



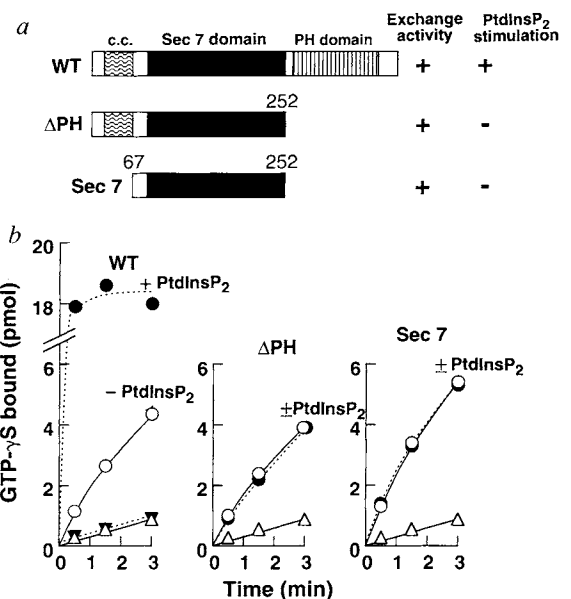
ARNO with PC vesicles with (+) or without (-) 30% PG, and/or 5% PtdInsP<sub>2</sub>. The binding of ARNO on vesicles of (PC + 30% PG + 5% PtdInsP<sub>2</sub>) was also measured at three different lipid concentrations of 0.1, 0.3 and 1 mg ml<sup>-1</sup>.

pase C $\delta$ , or spectrin (Fig. 3a). Because PH domains can interact with phosphatidylinositol bisphosphate (PtdInsP<sub>2</sub>)<sup>13,14</sup>, we tested the interaction of ARNO with PtdInsP<sub>2</sub> by replacing azolectin vesicles (Fig. 2) with vesicles of defined phospholipid composition. The exchange activity of ARNO is greatly stimulated when increasing amounts of PtdInsP<sub>2</sub> (up to 5%) are added to PC/PG vesicles (Fig. 3b); a control without ARNO shows that this addition of 5% PtdInsP<sub>2</sub> has no effect on the intrinsic exchange rate of ARF. With ARNO the effect of 5% PtdInsP<sub>2</sub> is hardly detectable when it is added to pure PC vesicles but remains high when 30% PG is replaced by 30% PS (Fig. 3c). Thus both PtdInsP<sub>2</sub> and a phospholipid with a single negative charge are required for maximal activity. Addition of 5% PtdInsP (or PtdIns) to PC/PG vesicles results in only a small stimulation of ARNO (PtdInsP being slightly more potent than PtdIns). PtdInsP<sub>2</sub> is the most potent compound tested, but we cannot exclude the possibility that other polyphosphoinositides such as PtdInsP<sub>3</sub> could be better activators. Stimulation by PtdInsP<sub>2</sub> is correlated with binding of the protein to PtdInsP<sub>2</sub>-containing vesicles, as demonstrated by sedimentation assay (Fig. 3d). Again, a negatively charged phospholipid (PG) is required for optimal interaction. The fraction of bound ARNO increases with lipid concentration (Fig. 3d), 67% of the protein being bound on PC/PG/PtdInsP<sub>2</sub> (65:30:5) vesicles at a lipid concentration of 1 mg ml<sup>-1</sup>, corresponding to ~50  $\mu$ M PtdInsP<sub>2</sub>. The PH domain of phospholipase C $\delta$  has a high affinity for PtdInsP<sub>2</sub> (~100 nM)<sup>13</sup>; other PH domains have affinities in the 10–100  $\mu$ M range<sup>14</sup>, so the PH domain of ARNO is in this second category.  $\Delta$ PH, a deletion mutant without the PH domain, can still stimulate exchange, but this activity is no longer stimulated by

PtdInsP<sub>2</sub> (Fig. 4), indicating that the PH domain must be indeed responsible for the interaction with PtdInsP<sub>2</sub>.

A mutant that is reduced to just the Sec7 domain stimulates exchange as efficiently as the  $\Delta$ PH mutant (Fig. 4), indicating that this domain is responsible for the exchange activity, whereas the PH domain mediates binding to PtdInsP<sub>2</sub> and presumably positions the exchange factor towards its membrane-bound target. A cluster of positively charged residues, C-terminal to the PH domain, may interact with negatively charged lipids and stabilize the association of the PH domain with PtdInsP<sub>2</sub>. PH domains are also found in exchange factors for other small GTP-binding proteins of the Dbp/Cdc24 family acting on Rho/Rac/Cdc42, or the Sos/rasGRF/ralGRF/C3G family acting on Ras/Ral/Rap. Sos is more active on the farnesylated version of ras<sup>15</sup>, and the PH

**FIG. 4** Effects of deletions on ARNO exchange activity and its stimulation by PtdInsP<sub>2</sub>. **a**, Deletion mutants of the PH domain ( $\Delta$ PH), or of the PH domain and coiled-coil (c.c.) region (Sec7 domain), and summary of the results. **b**, These truncated proteins were expressed in bacteria, purified and their exchange activity on ARF was tested as for Fig. 3, in the presence of PC/PG vesicles, supplemented with (▼, ●) or without (Δ, ○) 5% PtdInsP<sub>2</sub>. ▼, Δ, ARF only; ○, ●, ARF + ARNO (0.5  $\mu$ M of wild type (WT), or 75 nM of  $\Delta$ PH or of Sec7 domain).





domain of Dbl is needed for its activity *in vivo* but not in solution<sup>16</sup>, suggesting that the PH domains of Sos and Dbl also contribute to the positioning of these exchange factors on membranes.

ARNO belongs to a family that contains at least one closely related human homologue<sup>12</sup>, but partial sequences for more distantly related members are also found in EST databases. Additional proteins with Sec7 domains are present in *C. elegans*, *Arabidopsis* and yeast. Some of them are involved in vesicular traffic, but their precise function is still unknown. They could regulate other members of the ARF family.

ARF must be in the GTP-bound form to recruit coatamer to Golgi membranes and promote vesicle budding. It is still not clear whether it acts in a stoichiometric fashion as a binding site for one of the coatamer subunits or catalytically as an activator of a Golgi-localized phospholipase D, hydrolysing phosphatidylcholine to phosphatidic acid<sup>17</sup>, which could help coatamer binding to membranes<sup>1,2</sup>. Once ARNO is characterized, the mechanism of vesicle coating and the PtdInsP<sub>2</sub>-dependent regulation of phospholipase D should become clearer. □

## Methods

**Cloning and expression.** We searched for human homologues of the yeast ARF exchange factor Gea1 in the EST database. Several overlapping clones partly sequenced in the Merck–Washington University EST project<sup>9</sup> shared homology with Gea1 in the Sec7 domain. The largest clone (IMAGE clone ID, 180774; Genbank accession number, H52019; Research Genetics) was sequenced. The ARNO cDNA has a unique *Nco*I site at the ATG and a *Bam*HI site in the 3' non-coding region, and this *Nco*I–*Bam*HI fragment was cloned in the pET11d vector (Novagen). The full-length, non-fusion protein of 47K was expressed in *E. coli* grown at 35 °C in a 5-litre fermentor, induced with 0.1 mM IPTG. About 20 g of cells were lysed in 100 ml buffer A (50 mM Tris, pH 8, 1 mM DTT) with one tablet of Complete (Boehringer) protease inhibitors, essentially as described<sup>18</sup>. The soluble extract was loaded on a QAE–Sephacrose fast-flow column (~200 ml; Pharmacia) equilibrated in buffer A with 5 mM MgCl<sub>2</sub>, and eluted with a linear gradient of 0–500 mM NaCl (500 ml); pooled fractions containing ARNO (eluting at ~250 mM NaCl) were precipitated by ammonium sulphate (50% saturated), resuspended in 5 ml of buffer A plus 100 mM NaCl and loaded onto a gel-filtration column (~1 litre; Sephacryl S-100 HR, from Pharmacia) run in the same buffer; the major peak containing ARNO was concentrated by ammonium sulphate precipitation (60% saturated) and dialysed. The purified protein represents about 70% of the total protein content (1.7 mg ml<sup>-1</sup>; total of ~5 mg).

**Vesicle preparation.** A film of the desired lipids (PC, phosphatidyl choline; PG, phosphatidyl glycerol; PS, phosphatidyl serine; PtdInsP<sub>2</sub>, phosphatidylinositol biphosphate) was formed in a Rotavapor and resuspended in 50 mM HEPES buffer, pH 7.5, with 100 mM KCl. The suspension was vortexed for 20 min and freeze-thawed 5 times. Unilamellar vesicles used in exchange assays were produced by extrusion through 0.1-µm polycarbonate filters<sup>19</sup>. For sedimentation assays, a similar procedure was used, except that the buffer was 220 mM sucrose, 20 mM Tris, pH 7.5, and the extrusion was done through 0.4-µm polycarbonate filters. Sucrose-loaded vesicles were then diluted 5 times in 20 mM Tris, pH 7.5, 120 mM NaCl, centrifuged for 10 min at 400,000g, and resuspended in the same buffer.

**Exchange assays.** The exchange of GDP by [<sup>35</sup>S]GTP-γS on ARF was assayed at 37 °C essentially as described<sup>9</sup>, in the presence of azolectin vesicles (1.5 g l<sup>-1</sup>) or other vesicles of defined composition, with 1 mM MgCl<sub>2</sub> and 100 mM KCl. Samples of 25 µl were filtered at the indicated times. The ARF protein used was recombinant, fully myristoylated ARF1 (ref. 3). The lack of BFA effect was also evident on the crude bacterial extract.

**Lipid binding assay.** 1 µM ARNO was incubated with sucrose-loaded large unilamellar vesicles containing PC, PG and PtdInsP<sub>2</sub> in the indicated proportions. After 10 min, the suspension was centrifuged at 400,000g for 10 min and the fraction of ARNO in the pellet determined by SDS–PAGE.

**Preparation of deletion mutants.** Fragments were generated by polymerase chain reaction (PCR) using Vent DNA polymerase (Biolabs), cloned in pET11 vectors, and the sequences checked. Proteins were purified as described for wild-type ARNO, with minor modifications. ΔPH has an apparent *M<sub>r</sub>* of 80K on gel filtration and is probably dimeric like wild-type ARNO, but Sec7 has an apparent *M<sub>r</sub>* of 30K and is probably monomeric.

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## Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation

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**REGULATION through phosphorylation is a characteristic of signalling pathways<sup>1,2</sup>, and the lymphocyte kinase Lck (p56<sup>lck</sup>) both performs phosphorylation and is affected by it. Lck is a Src-family tyrosine kinase expressed in T lymphocytes, where it participates in the cellular immune response<sup>3</sup>. Like all Src homologues, it comprises SH3, SH2 and kinase domains. Lck associates through its distinctive amino-terminal segment with the cytoplasmic tails of either T-cell co-receptor, CD4 or CD8-α<sup>4,5</sup>. Activated Lck phosphorylates T-cell receptor ζ-chains, which then recruit the ZAP70 kinase to promote T-cell activation<sup>6</sup>. Lck is activated by autophosphorylation at Tyr 394 in the activation loop<sup>7</sup> and it is inactive when Tyr 505 near the carboxy terminus is phosphorylated and interacts with its own SH2 domain<sup>8</sup>. Here we report the crystal structure of the Lck tyrosine kinase domain (LCKK) in its activated state at 1.7 Å resolution. The structure reveals how a phosphoryl group at Tyr 394 generates a competent active site. Comparisons with other kinase structures indicate that tyrosine phosphorylation and ligand binding may in general elicit two distinct hinge-like movements between the kinase subdomains. From modelling studies, we suggest a basis for inhibition by phosphorylation at Tyr 505.**

We produced and specifically phosphorylated a construct of the kinase domain of human Lck (LCKK), residues 225–509. The crystal structure was determined by a combination of phasing methods, and the atomic model has been refined at 1.7-Å resolution to an *R*-value of 17.8% from a crystal of LCKK phosphorylated exclusively at Tyr 394 and grown in the absence of substrates (Table 1). The structure is generally well defined (Fig. 1a) for the ordered portion (residues 231–501), with an average *B*-factor of 8.5 Å<sup>2</sup>, but parts of the activation loop have higher *B*-factors and the electron density is feeble at positions 391–392 and 397–399.

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TABLE 1 Crystallographic analysis

| Diffraction data                                                         |                                     |                    |                      |                      |                            |                      |                  |                  |                  |
|--------------------------------------------------------------------------|-------------------------------------|--------------------|----------------------|----------------------|----------------------------|----------------------|------------------|------------------|------------------|
| Data set                                                                 |                                     | Wavelength<br>(Å)  | Resolution<br>(Å)    | Reflections*         | Redundancy†                | Completeness‡<br>(%) | Rsym‡§<br>(%)    |                  |                  |
| Native                                                                   | 1                                   | 1.02298            | 20.0–1.7             | 31,176               | 6.7                        | 97.7<br>(92.3)       | 4.2<br>(11.6)    |                  |                  |
|                                                                          | 2                                   | 0.97950            | 20.0–1.7             | 31,599               | 8.1                        | 99.0<br>(96.1)       | 2.8<br>(4.7)     |                  |                  |
| MIR                                                                      | K <sub>2</sub> PtCl <sub>4</sub> –1 | 0.96412            | 20.0–2.0             | 18,975               | 5.4                        | 95.9<br>(74.0)       | 2.7<br>(7.0)     |                  |                  |
|                                                                          | K <sub>2</sub> PtCl <sub>4</sub> –2 | 1.07180            | 20.0–2.0             | 19,450               | 6.6                        | 98.3<br>(93.4)       | 4.2<br>(9.7)     |                  |                  |
|                                                                          |                                     | 1.07217            | 20.0–2.0             | 19,473               | 6.6                        | 98.4<br>(94.5)       | 4.9<br>(12.4)    |                  |                  |
| MAD                                                                      | K <sub>2</sub> PtCl <sub>4</sub> –3 | 1.05790            | 20.0–2.0             | 17,933               | 3.4                        | 72.7<br>(35.6)       | 6.1<br>(11.0)    |                  |                  |
|                                                                          |                                     | 1.07161            | 20.0–2.0             | 18,129               | 3.4                        | 72.1<br>(32.5)       | 6.4<br>(9.6)     |                  |                  |
|                                                                          |                                     | 1.07212            | 20.0–2.0             | 18,102               | 3.4                        | 71.8<br>(32.0)       | 5.9<br>(9.3)     |                  |                  |
|                                                                          |                                     | 1.08569            | 20.0–2.0             | 18,263               | 3.3                        | 70.9<br>(28.8)       | 5.5<br>(10.2)    |                  |                  |
| Phasing                                                                  |                                     |                    |                      |                      |                            |                      |                  |                  |                  |
| MIR (against native-1)¶                                                  |                                     | Sites              | R <sub>iso</sub> (%) | R <sub>cen</sub> (%) | Phasing power              |                      |                  |                  |                  |
| K <sub>2</sub> PtCl <sub>4</sub> –1                                      |                                     | 5                  | 23.5                 | 72                   | 1.8/1.2                    |                      |                  |                  |                  |
| K <sub>2</sub> PtCl <sub>4</sub> –2 (λ.1)                                |                                     | 4                  | 20.4                 | 71                   | 1.9/1.2                    |                      |                  |                  |                  |
| K <sub>2</sub> PtCl <sub>4</sub> –2 (λ.2)                                |                                     |                    | 15.9                 | 79                   | 1.6/1.0                    |                      |                  |                  |                  |
| MAD¶ structure factor ratio                                              |                                     |                    |                      |                      |                            |                      |                  |                  |                  |
|                                                                          |                                     | 20.0 Å < d < 3.0 Å |                      |                      |                            | 3.0 Å < d < 2.0 Å    |                  |                  |                  |
| Wavelength (Å)                                                           |                                     | λ.1                | λ.2                  | λ.3                  | λ.4                        | λ.1                  | λ.2              | λ.3              | λ.4              |
| λ.1(1.05790)                                                             |                                     | 0.049<br>(0.038)   | 0.027                | 0.031                | 0.030                      | 0.070<br>(0.054)     | 0.048            | 0.048            | 0.049            |
| λ.2(1.07161)                                                             |                                     |                    | 0.053<br>(0.038)     | 0.023                | 0.028                      |                      | 0.075<br>(0.053) | 0.045            | 0.048            |
| λ.3(1.07212)                                                             |                                     |                    |                      | 0.049<br>(0.041)     | 0.031                      |                      |                  | 0.069<br>(0.062) | 0.049            |
| λ.4(1.08569)                                                             |                                     |                    |                      |                      | 0.039<br>(0.037)           |                      |                  |                  | 0.060<br>(0.059) |
| Sites = 5                                                                |                                     |                    |                      |                      |                            |                      |                  |                  |                  |
| R(  <sup>o</sup> F <sub>i</sub>  ) = 0.064                               |                                     | ⟨Δ(Δϕ)⟩ =          |                      | 23.4°                |                            |                      |                  |                  |                  |
| R(  <sup>o</sup> F <sub>a</sub>  ) = 0.409                               |                                     | ⟨σ(Δϕ)⟩ =          |                      | 19.8°                |                            |                      |                  |                  |                  |
| ⟨f.o.m.⟩ = 0.50                                                          |                                     |                    |                      |                      |                            |                      |                  |                  |                  |
| (c) Refinement (against native-2)#                                       |                                     |                    |                      |                      |                            |                      |                  |                  |                  |
| Model: 271 amino-acid residues, 340 water molecules, 1 sulphate molecule |                                     |                    |                      |                      |                            |                      |                  |                  |                  |
|                                                                          |                                     |                    |                      | R.m.s. deviations    |                            |                      |                  |                  |                  |
| Resolution (Å)                                                           | Reflections (N)                     | R-value (%)        | Bonds (Å)            | Angles (°)           | B-values (Å <sup>2</sup> ) |                      |                  |                  |                  |
| 10.0–1.7                                                                 | 31,241                              | 17.8               | 0.005                | 1.90                 | 1.22                       |                      |                  |                  |                  |

\* For the native and MIR data sets, unique reflections were counted in point group *Pmmm*; for the MAD data set, acentrics in point group *P222* and centrics in *Pmmm*.

† Values for redundancy represent ratios of the total number of measurements to the number of unique reflections.

‡ Values in parentheses are for the last resolution shell; 1.76–1.70 Å for native data sets, 2.05–2.00 Å for MIR data sets, or 2.07–2.00 Å for MAD data set.

§  $R_{\text{sym}} = 100 \times \sum_{hkl} \sum_i |I_i - \langle I \rangle| / \sum_{hkl} \sum_i I_i$ , where  $I_i$  is the  $i$ th measurement and  $\langle I \rangle$  is the weighted mean of all measurements of  $I$ .

||  $R_{\text{iso}} = 100 \times \sum_i ||F_{\text{ph}}| - |F_{\text{p}}|| / \sum_i |F_{\text{p}}|$ , where  $|F_{\text{ph}}|$  and  $|F_{\text{p}}|$  are heavy-atom derivative and protein structure factor amplitudes, respectively;  
 $R_{\text{cen}} = 100 \times \sum_i ||F_{\text{ph}} \pm F_{\text{p}}| - F_{\text{calc}}| / \sum_i |F_{\text{ph}} - F_{\text{p}}|$  for centric reflections; and phasing power =  $\langle F_{\text{h}} \rangle / E$ , where  $\langle F_{\text{h}} \rangle$  is the mean of calculated heavy-atom structure factor amplitudes; and  $E$  is the r.m.s. lack of closure error.

¶ Table values for structure factor ratios represent r.m.s. ( $\Delta|F|$ )/r.m.s. ( $|F|$ ), where  $\Delta|F|$  is the Bijvoet difference at one wavelength (diagonal elements) or the dispersive difference between two wavelengths (off-diagonal elements). The values in parentheses are the ratios for centric Bijvoet reflections, which would be equal to zero for perfect data and serve as an estimate of the noise in the anomalous signals.  $R = \sum_h \sum_i |F_i(h) - \langle F(h) \rangle|$ .  $^{\circ}F_i$  is the structure factor due to normal scattering from all the atoms,  $^{\circ}F_a$  is the structure factor due to normal scattering from the anomalous scatterers only, and  $\Delta\phi$  is the phase difference between  $^{\circ}F_i$  and  $^{\circ}F_a$ .  $\Delta(\Delta\phi)$  is the difference between two independent determinations of  $\Delta\phi$ . Values given are based on calculations that did not include reflections with f.o.m. = 0; (f.o.m.) is the mean figure of merit including reflections with f.o.m. = 0.

# A subset of the data (10%) was excluded from the refinement and used for the free  $R$ -value calculation. The final round of refinement was carried out with all of the data ( $F > 2\sigma$ ).  $R$ -value =  $100 \times \sum ||F_{\text{o}}| - |F_{\text{c}}|| / \sum |F_{\text{o}}|$ . r.m.s. deviation in  $B$ -values is for bonded atoms.

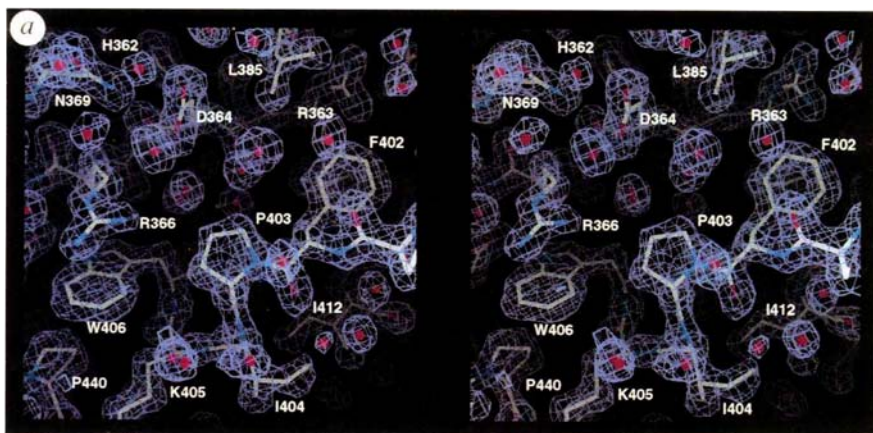
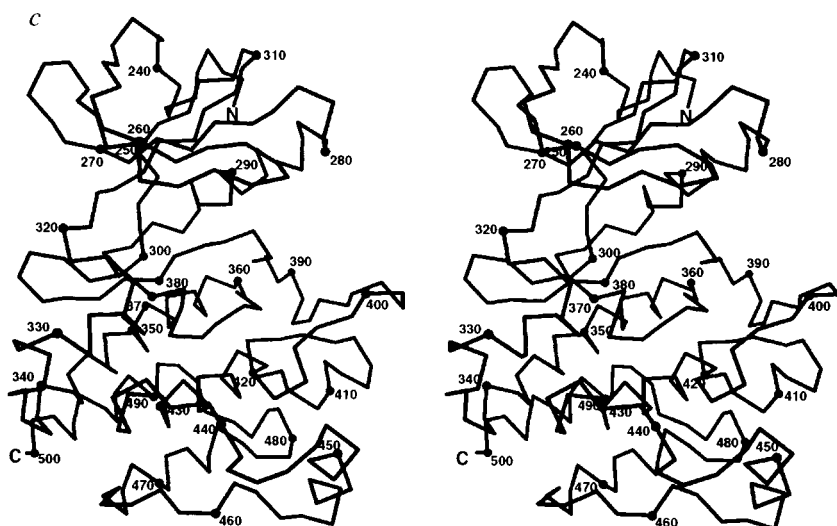
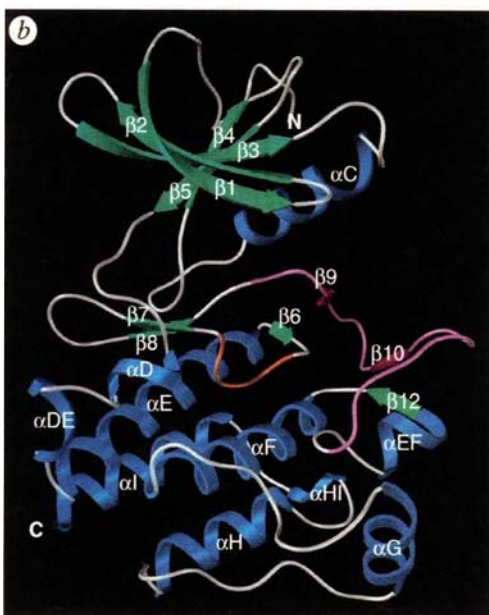


FIG. 1 *a*, Stereo view of the electron-density map in the vicinity of the catalytic loop and P + 1 loop. The Fourier transformation used phases calculated from the model and  $2F_o - F_c$  amplitudes with  $F_o$  values from native data set 2. Reflections for 10.0–1.7 Å spacings were included. Contours are at  $1.3\sigma$ . *b*, Ribbon diagram of the kinase domain of human LCK. The  $\alpha$ -helices are shown in blue, the  $\beta$ -strands in green, the catalytic loop in orange, and the activation loop and P + 1 loop in magenta. The termini are labelled as N and C. *c*, Stereo view of C $\alpha$  trace of LCKK. The orientation is the same as in *b*. Every tenth  $\alpha$ -carbon is labelled with its residue number, and both termini are labelled as N and C. The whole figure was prepared with SETOR<sup>27</sup>.



As expected, the structure is similar in folding to other protein kinase domains (Fig. 1*b*, *c*), and we adopt the established nomenclature for structural elements<sup>9,10</sup> (Fig. 1*b*).

We have compared this LCKK structure with those of the unphosphorylated insulin-receptor tyrosine kinase domain, IRK (Protein Database (PDB) entry 1IRK)<sup>9</sup>, and of cAMP-dependent kinase as a ternary complex, cAPK (PDB entry 1ATP), and in the unligated apo state, cAPK<sub>apo</sub><sup>11</sup> (J. Sowadski, personal communication); structures are not yet known for LCKK in these other relevant states. Superpositions of the individual subdomains show that LCKK is slightly more similar to IRK than to cAPK, in accord with the levels of sequence similarity (Table 2). However, there are some large differences in the relative disposition of the two subdomains among the three protein kinases and in the conformation of the activation loop between LCKK and IRK. Other structural differences between LCKK and IRK are found in both termini, in the glycine-rich loop, in the loop connecting  $\beta 2$  and  $\beta 3$ , in the kinase insert region which includes a new helix  $\alpha DE$ , and in the position of  $\alpha G$  and the loops connecting it to  $\alpha F$  and  $\alpha H$ . The differences at the glycine-rich loop, and perhaps at  $\alpha G$ , seem to be connected to the large change in activation-loop conformation.

The activation loop of LCKK has a radically different conformation from that of the corresponding unphosphorylated loop of

IRK (Fig. 2*a*). These loops diverge after Ala 381 (1,149 in IRK) and reconverge at Phe 402 (1,171 in IRK) with displacements of C $\alpha$  atoms as large as 31.2 Å (391 versus 1,159). The activation loop in the unphosphorylated kinase from fibroblast growth-factor receptor 1 (ref. 12) differs markedly from that in either IRK or LCKK. In contrast, the phosphorylated activation loops of LCKK and cAPK have generally similar conformations (Fig. 2*a*) through the site of phosphorylation (displacements greater than 2.5 Å only at residues 390–391 of LCKK, where there is an insertion relative to 192 of cAPK), but they diverge more into the substrate-binding P + 1 loop (displacements exceed 2.5 Å between Ala 396 (Thr 197 in cAPK) and Lys 405 (203 in cAPK), with the largest being 6.1 Å (401 versus 199)). This latter distinction between LCKK and cAPK probably relates to the corresponding specificity for tyrosine versus serine/threonine phosphorylation<sup>9</sup>. Phosphotyrosine (pTyr) 394 is on strand  $\beta 10$ , which is orientated in part by  $\beta 12$ , newly defined in LCKK but of the same conformation as in IRK. The residues on either side of  $\beta 10$  are highly mobile relative to the rest of LCKK.

Backbone positions of the phosphorylated residues in the activation loop are displaced between LCKK and cAPK (pTyr 394 corresponds to Thr 195 in cAPK rather than to pThr 197), but the phosphoryl groups occupy nearly equivalent sites (2.8 Å

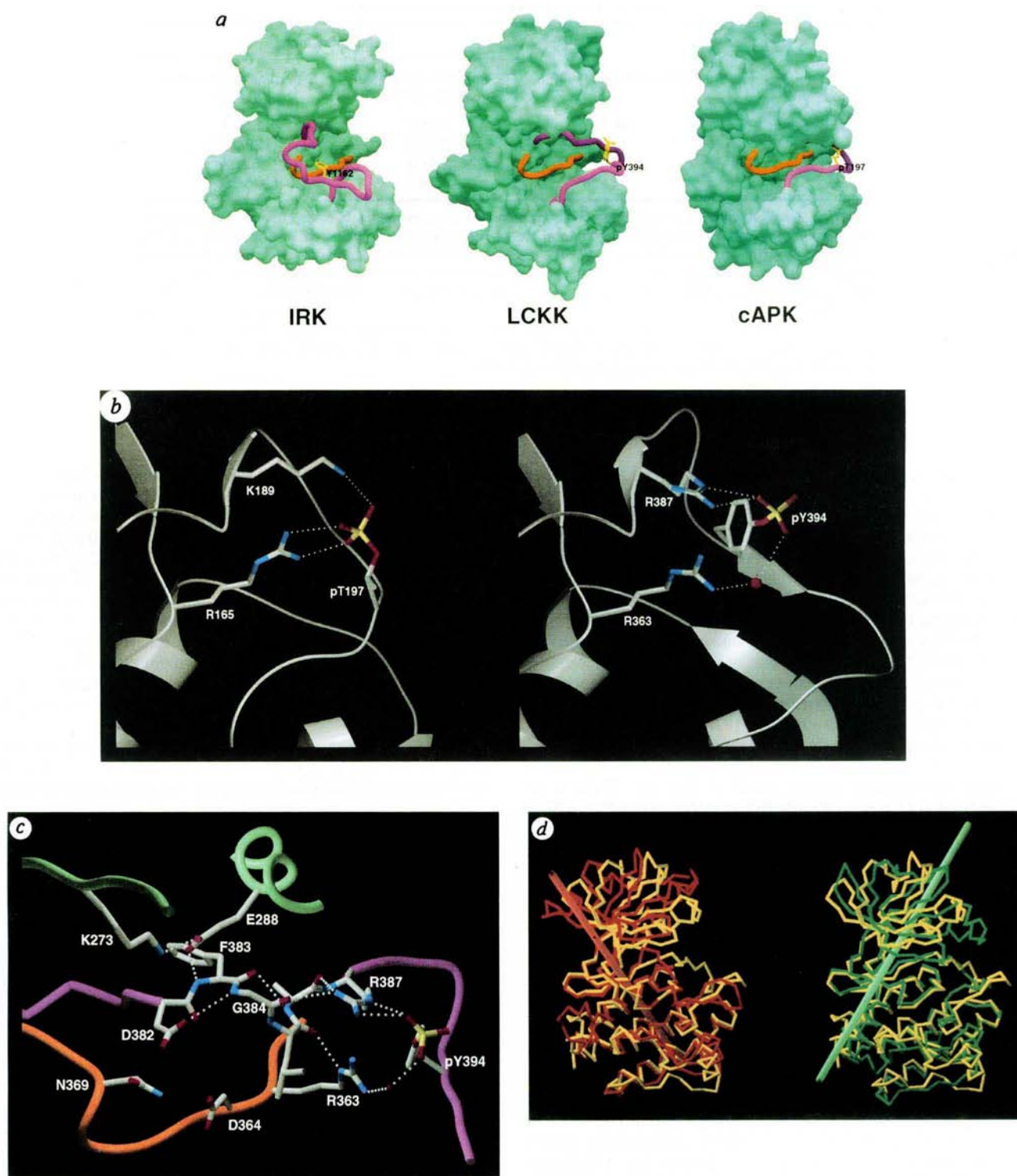


FIG. 2 *a*, Surface representation of IRK, LCKK and cAPK. The catalytic loops are shown in orange, and the activation and P + 1 loops in magenta. The residues 1,162 in IRK, 394 in LCKK, and 197 in cAPK, are shown in yellow. The orientations are similar to those in Fig. 5(A) of ref. 12. Prepared using GRASP<sup>28</sup>. *b*, Ribbon diagrams of activation loops for cAPK (left) and LCKK (right). Ionic interactions are shown by dotted lines between the phosphate group and positively charged side chains. A coordinating water molecule in LCKK is shown as a dark pink sphere. Prepared using SETOR<sup>27</sup>. *c*, Schematic diagram of interactions relaying from phosphate group on Tyr 394 to the catalytic centre of LCKK. Parts of  $\beta 3$  and  $\alpha C$  from N-terminal subdomain are shown in green, the activation loop in magenta, and the catalytic loop in

orange. Ionic interactions are shown by dotted lines. Prepared using SETOR<sup>27</sup>. *d*, Superpositions of C $\alpha$  traces between LCKK (231–501) and IRK (981–1266) (left), and between LCKK (231–501) and cAPK (35–294) (right). IRK is shown in red, cAPK in green, and LCKK in yellow. The superposition was done in two steps; first, C $\alpha$  atoms in the catalytic loop were superimposed, then the superposition was extended to the entire carboxy-terminal subdomain by including only the paired C $\alpha$  atoms that are within 2.5 Å in segments of at least three residues. The axes of rotation required for the superpositions of N-terminal subdomains alone are shown as thick rods. Prepared with SETOR<sup>27</sup>.



TABLE 2 Kinase domain structural comparisons

|                  | Sequence identity (%) | $\alpha$ superpositions           |                                   |
|------------------|-----------------------|-----------------------------------|-----------------------------------|
|                  |                       | N-terminal subdomain<br>(236–321) | C-terminal subdomain<br>(320–501) |
| LCKK versus IRK  | 38.1                  | 1.09 Å<br>(77/86)                 | 1.04 Å<br>(128/182)               |
| LCKK versus cAPK | 18.9                  | 1.18 Å<br>(63/86)                 | 1.20 Å<br>(106/182)               |

R.m.s. deviations of  $\alpha$  atoms for superpositions of individual subdomains between LCKK and IRK or cAPK. The number of paired  $\alpha$  atoms ( $M$ ) and the total number of LCKK residues within each subdomain ( $N$ ) in the superposition are given in parentheses as  $M/N$ . The residue ranges of N- and C-terminal subdomains are defined for LCKK as shown in the table. The superpositions were done in two steps; first, entire structures were aligned with each other by superposing the  $\alpha$  atoms in the C-terminal subdomains, then  $\alpha$  atoms in the N-terminal subdomain were superposed. The alignment of the C-terminal subdomain was initially done only with the catalytic loop, then extended to the entire subdomain. With the distance cut-off criteria (2.5 Å apart in at least 3 contiguous residues) used, the  $\alpha$ G and  $\alpha$ L helices were excluded in the case of LCKK versus cAPK, and the activation loop and  $\alpha$ G helix in the case of LCKK versus IRK.

apart). Ionic interactions of the phosphoryl groups are therefore similar in the two enzymes (Fig. 2b). Each interacts with one positively charged side chain from upstream in the activation loop (Lys 189 in cAPK, Arg 387 in LCKK) and another from the catalytic loop (Arg 165 in cAPK, Arg 363 in LCKK). Although these are conserved residues, their roles in phosphate coordination are interchanged. In cAPK, Arg 165 plays the major role with two hydrogen bonds, whereas Lys 189 forms a single hydrogen bond. In the case of LCKK, Arg 363 makes the smaller contribution by interacting through a water molecule, whereas Arg 387 makes two hydrogen bonds. Other parts of the activation loop also contribute to phosphate coordination in each case, but these are not in common. Thr 195O $\gamma$  and Thr 197N each form a hydrogen bond directly to a phosphate oxygen atom in cAPK, but in LCKK there are two water-mediated hydrogen bonds from the amide nitrogen and carbonyl oxygen atoms of Thr 395. There is no counterpart in LCKK for the phosphate interaction with His 87 in cAPK or with Arg 50 of Cdk2 complexed with cyclin A<sup>13</sup>. The phosphate group of pTyr 394 in LCKK is anchored rather weakly, as indicated by its relatively high  $B$ -factors ( $\sim 40$  Å<sup>2</sup>). The elevated mobility here and in nearby activation-loop segments may facilitate the regulatory deactivation by phosphatases that is typical for tyrosine kinases<sup>14</sup>. By contrast, the phosphate group of pThr 197 of cAPK is normally always present<sup>15</sup>; once activated by phosphorylation, cAPK uses a regulatory subunit to suppress the activity<sup>16</sup>. Moreover, the structural effects of threonine phosphorylation of Cdk2 are local<sup>13</sup>, whereas tyrosine phosphorylation appears to involve large-scale changes.

How does phosphorylation promote tyrosine kinase activation? One way is through the removal of autoinhibition<sup>9–12</sup>. Here we also see direct activation by the phosphoryl group. In LCKK there is an intricate and quite direct relay of interactions from pTyr 394 into the enzymatic centre (Fig. 2c). Arginyl guanidinium groups mediate hydrogen-bonding bridges between the phosphoryl group and carbonyl oxygen atoms, through Arg 387 to Gly 384O and Ala 386O, and through Arg 363 of the catalytic loop to Leu 385O. Hydrophobic contacts between pTyr 394C $\beta$  and Ile 361 and Ile 389, and between Leu 385 and Arg 363C $\beta$  and Asp 364C $\beta$ , buttress these interactions. A  $3_{10}$ -helical backbone hydrogen bond between Ala 386N and Phe 383O, and another from Gly 384N to the carboxyl side chain of Asp 382, then specify the conformation of the absolutely conserved Asp-Phe-Gly loop. This is such that the carboxyl group of Glu 288 bridges the subdomains with hydrogen bonds to both Phe 383N and Lys 273N $\zeta$ . In addition, and as in cAPK but not IRK, there are hydrophobic contacts from Phe 383 (185 in cAPK) and Ala 385 (187) to Leu 291 (94) and Met 292. Ultimately, the catalytically important residues Lys 273 (72),

Glu 288 (90), Asp 382 (184), Asp 364 (162) and Asn 369 (167) are all in equivalent positions in LCKK and cAPK, but are disposed very differently in IRK (root-mean-square deviations between  $\alpha$  positions for these residues range from 0.61 to 0.83 Å among LCKK, cAPK (ternary) and cAPK<sub>apo</sub>, and from 2.16 to 2.45 Å from IRK to the others). Thus the disposition of these five residues seems to characterize the activated state. In the place of ATP and peptide substrates, an elaborate network of water molecules occupies the active site of LCKK (Fig. 1a). Only the glycine-rich loop, which must close down onto the substrate after initial binding, as occurs between the apo and ternary complex states of cAPK, is not in the ultimate position for an ATP complex.

The conformation of a particular state of a kinase has been characterized by the openness of the ATP-binding cleft at the subdomain interface. Conformational openness seems to corre-

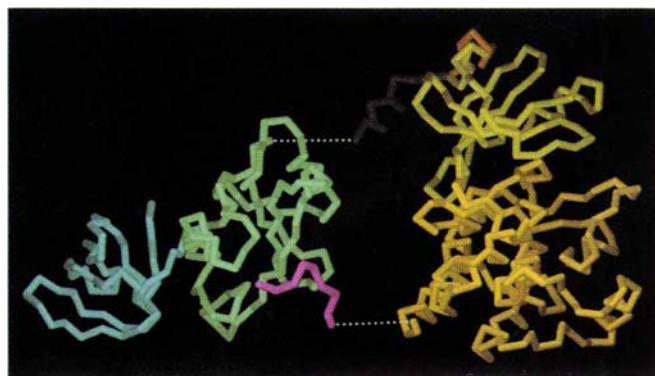


FIG. 3 Hypothetical intramolecular complex between the C-terminal extension and SH2 domain of Lck. The SH3–SH2–phosphopeptide complex, taken from ref. 20, has been docked onto LCKK. The  $\alpha$  trace of this model is shown with the components separated as indicated by dotted lines. The SH3 domain is shown in cyan, the SH2 domain in green, the phospho-peptide corresponding to the carboxy-terminal tail of the kinase domain (502–509) in magenta, the N-terminal subdomain of LCKK in greenish yellow, and the C-terminal subdomain in dark yellow. The segment of residues 226–237 that connects the SH2 and kinase domains is shown in grey in one of several conformations that meets distance constraints. Residues 231–237 have been refolded from their structure in LCKK, in orange. Prepared with SETOR<sup>27</sup>.

late with the state of the enzyme (for example, whether it is activated by phosphorylation and/or complexed with ligands<sup>2,17,18</sup>), although seemingly irrelevant factors such as crystal packing may sometimes have an influence<sup>17</sup>. The kinases that we chose for comparison are in states that share certain features, but not others, with LCKK; IRK is also a ligand-free tyrosine kinase but is unphosphorylated, whereas cAPK is also phosphorylated on the activation loop but is complexed with ligands. With common elements of the C-terminal subdomains superimposed (Table 2), the transformation required to superimpose the N-terminal subdomains was analysed. The rotation needed to place LCKK onto IRK is 17°, and that onto cAPK is 11°; the translational components are negligible at less than 0.5 Å. The rotation relating LCKK and cAPK<sub>apo</sub> subdomains is just 4°. The LCKK conformation is intermediate between the other two (Fig. 2d), but the two transformation axes are nearly orthogonal (75.7°). For IRK versus LCKK it passes close to Tyr 318, and is nearly perpendicular to the plane that separates the two subdomains; but for cAPK versus LCKK it passes nearest to Ser 323 and is almost parallel to the inter-subdomain plane, this is also true for cAPK(ternary) versus cAPK<sub>apo</sub>. Because we have necessarily compared different states from different proteins, the relevance and generality of these findings is untested. Nevertheless, we suggest that two such distinct rotations may pertain quite broadly for tyrosine kinases, first in the activating transition to form a competent binding site upon phosphorylation, and then in the induced-fit process upon binding of ligands. Verification of this hypothesis awaits the structure of LCKK in both the non-phosphorylated and the ligand-bound states.

The binding of pTyr 505 from the C-terminal tail of Lck to its own SH2 domain suppresses kinase activity, and such a regulatory mechanism is thought to be general for Src kinases<sup>19</sup>. In attempting to dock the known SH2 structure of Lck<sup>20</sup> onto LCKK, we found that an intramolecular complex is impossible if both structures stay unchanged. Large-scale rearrangements of SH2 seem unlikely, but the conformation of residues 231–235 in LCKK is quite different from the counterpart segment of IRK, and both Trp 233 and Trp 234 are exposed to lattice contacts in the crystal. After this segment is unfolded and extended to connect the two domains, the SH2–pTyr 505 complex can be docked onto the 'back side' of LCKK (Fig. 3). Only if helix  $\alpha$ 1 were unfolded, not even if the entire  $\beta$ 1 strand were peeled back as in the p27–Cdk2-cyclin complex<sup>21</sup>, could the SH2 domain reach the front side to block the active site<sup>22</sup>. We suggest from this model that SH2 binding to pTyr 505 deprives the kinase domain of the freedom to make one or both of the domain-closing movements required for catalytic activity. □

## Methods

**Expression, purification and crystallization.** A recombinant baculovirus was constructed using the Bac-to-Bac system (Life Technologies) to insert a fragment from the cDNA of human *lck*<sup>3</sup> that encodes the kinase domain (residues 225–509) downstream of the polyhedrin promoter. The expressed protein was purified from the lysate of Sf9 cells infected by the recombinant virus on Source 15Q, Superdex 200, and Superdex 75 chromatographic columns at 4 °C. Partly purified material was treated with 1 mM Mg-ATP for 1 min at room temperature, and autophosphorylation was stopped in 5 mM EDTA. The reaction mixture was freed of nucleotides on a Superdex75 column, and the pTyr 394 LCKK was separated from other components on a Source 15Q column.

Initial crystals were obtained by vapour diffusion in hanging drops that contained equal volumes of the cocktail solution of 0.64 mg ml<sup>-1</sup> purified protein, 100  $\mu$ M ATP, and 100  $\mu$ M substrate peptide (EDYTKIEKIGETGVVKK) and the reservoir solution of 2.0 M ammonium sulphate and 0.1 M BisTris buffer, pH 6.5. Single needle-shaped crystals were macroseeded in a solution containing 0.51 mg ml<sup>-1</sup> protein, 80  $\mu$ M ATP, 80  $\mu$ M substrate peptide, 0.6 M ammonium sulphate and 0.05 M BisTris buffer, pH 6.5, and equilibrated against the reservoir solution of 1.6 M ammonium sulphate and 0.1 M BisTris buffer, pH 6.5. Several rounds of macroseeding were conducted to grow crystals to an average size of 0.06  $\times$  0.06  $\times$  0.7 mm. Both ATP and substrate peptide were omitted from the macroseeding condition for native crystal 2, but they were otherwise present.

**Data collection and processing.** Data were measured at HHMI beamline X4A at the National Synchrotron Light Source, Brookhaven National Laboratory, on Fuji imaging plates (IPs). Crystals cryoprotected by 20% ethylene glycol were frozen in a nitrogen gas stream at a temperature of 100 K. The crystals are in space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> with unit-cell dimensions of  $a = 42.04$  Å,  $b = 73.62$  Å,  $c = 91.17$  Å (native crystal 2), one protein molecule per asymmetric unit. Derivatives were prepared by soaking crystals in a solution containing 5 mM K<sub>2</sub>PtCl<sub>6</sub>, 100  $\mu$ M ATP, 100  $\mu$ M substrate peptide, 1.6 M ammonium sulphate and 0.1 M bis-Tris buffer, pH 6.5, at 20 °C for 20 h followed by back-soaking for 1 h in a platinum-free solution. Crystals were aligned for simultaneous measurement of Bijvoet mates to optimize anomalous signals. IP images were scanned with a Fuji scanner and processed into reflection intensities using the program DENZO (Z. Otinowski and W. Minor, unpublished). ROTAVATA<sup>23</sup> and AGROVATA<sup>23</sup> or SCALEPACK (Z. Otinowski and W. Minor, unpublished) were used to scale and merge data. The MAD data set was scaled but kept unmerged.

Molecular replacement was done in XPLOR<sup>24</sup> with the main-chain and C $\beta$  atoms in secondary structure segments of IRK. Multiple isomorphous replacement (MIR) phases were calculated by MLPHARE<sup>23</sup> after scaling by SCALEIT<sup>23</sup>. These phases were modified by solvent flattening in DM<sup>23</sup> and combined with the molecular replacement phases by SIGMAA<sup>23</sup>. These programs were modified to output Hendrickson-Lattman phase probability coefficients. Multiwavelength anomalous diffraction (MAD) phases were calculated by the MADSYS approach<sup>25</sup>. Multiwavelength anomalous diffraction (MAD) derived phase probability distributions for the derivative structure were geometrically transferred to those for the native structure, modified by DM, and combined with the molecular replacement- or MIR-derived phases by SIGMAA.

**Model building and refinement.** Atomic models were built with program O<sup>26</sup> on SGI workstations. An initial model was fitted to the electron-density map calculated from native data set 1 with the modified experimental phases. Phases calculated from intermediate models were recombined with the experimental phases for subsequent fitting. After the model was 72% complete, no significant additional electron-density features were found, and this model was refined against the 6.0 to 1.7 Å data of native data set 1 with X-PLOR by a simulated annealing protocol. The experimental phases were combined once more with the partial model phases, and the subsequent rounds of fitting were done with 2F<sub>o</sub>-F<sub>c</sub> maps. Several rounds of positional and B-factor refinement were done as the lower resolution limit was gradually extended down to 10.0 Å and water molecules were included. There was no sign of the substrate peptide or ATP in the 2F<sub>o</sub>-F<sub>c</sub> or F<sub>o</sub>-F<sub>c</sub> electron-density maps calculated from native data set 1. Refinement against the ligand-free native data set 2 showed that both structures were virtually identical and free of ligands.

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# Antibody characterization by isothermal titration calorimetry

Jeff R. Livingstone

**Isothermal titration calorimetry is a useful tool for the characterization of antibodies and antibody-related products, which uses heat as a detection medium, circumventing the problems associated with other analytical methods.**

PHYSICAL characterization of biological products represents an important issue in research, as well as pharmaceutical and biotechnology product development. In particular, antibodies and their products are sometimes characterized by the strength or weakness of their affinity to specific antigens. Techniques that provide measures of affinity constants include isothermal titration calorimetry (ITC)<sup>1</sup>, analytical ultracentrifugation<sup>2</sup>, fluorometry<sup>3</sup>, enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA)<sup>4</sup>, plasmon resonance methods (for example, BIAcore<sup>5</sup>), and equilibrium dialysis<sup>6</sup>. However, of those true 'in-solution' methods that do not require modification of the antibody or antigen (equilibrium, analytical ultracentrifugation and ITC), only ITC can provide the user with a

complete thermodynamic characterization of the system under study. ITC measurements typically can be accomplished in approximately 30–60 min, are non-destructive to the sample, and can be applied over a broad range of solution conditions. This article will review the information that can be derived from ITC and detail the role ITC plays in the biophysical characterization of antibodies and antibody products.

## The ITC experiment

ITC uses heat as a signal source. A syringe containing a solution of one element, commonly referred to as the 'ligand', is incrementally titrated into a cell containing a solution of the second element, commonly referred to as the 'macromolecule'. This is illustrated in Fig. 1, which contains a schematic of the ITC cell assembly. As the ligand is added to the macromolecule, heat is released or absorbed upon interaction of the two entities. The heat for each injection is measured by the ITC instrument and is plotted as a function of time over the injection series (Fig. 2a). As the macromolecule in the cell becomes saturated with ligand, the heat signal diminishes until at full saturation, where only background heat from the dilution of the ligand is observed.

In a typical analysis, the total heat signal from each injection is determined by the area underneath the injection peak. When this total heat is plotted against the molar ratio of ligand added to macromolecule in the cell, as demonstrated in Fig. 2b, one obtains a complete binding isotherm for the interaction of ligand and macromolecule. With instruments such as the Microcal MCS-ITC system, the entire ITC experiment, from the timing and control of the injections to the acquisition of data, is entirely under computer control. The user inputs the experimental

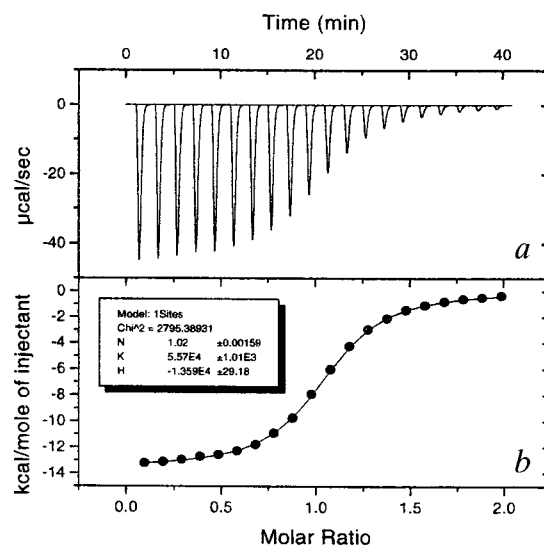


FIG. 2 Representative data from an ITC experiment. Panel *a* shows the raw heat data obtained over a series of injections. Panel *b* shows a binding isotherm created by plotting the areas under the peaks in panel *a* against the molar ratio of ligand added to macromolecule present in the cell. The box represents the fitted values for the stoichiometry, equilibrium constant, and enthalpy, respectively. The  $\chi^2$  statistic of the fit is given. The solid line represents a nonlinear fit using a 'one-set-of-sites' model.

parameters (temperature, number of injections, injection volumes) and the computer carries out the experiment. After acquisition, the system can analyse the raw ITC data by application of the appropriate binding model. For a complete overview of the analysis and interpretation of ITC results see the reviews by Bundle and Sigurskjold<sup>7</sup> and Fisher and Singh<sup>8</sup>.

Unlike other methods, ITC provides the user with a great deal of physical information about the binding process, typically from a single experiment. This information includes the binding affinity or binding equilibrium constant ( $K_{eq}$ ), the molecular ratio or binding stoichiometry ( $n$ ), and the heat or enthalpy ( $\Delta H$ ), as well as entropy ( $\Delta S$ ) of binding. The enthalpy of binding, stoichiometry, and free energy change ( $\Delta G$ ) of the binding process are determined by a nonlinear fit of the binding isotherm (Fig. 2b). The entropy of binding is determined from a difference between

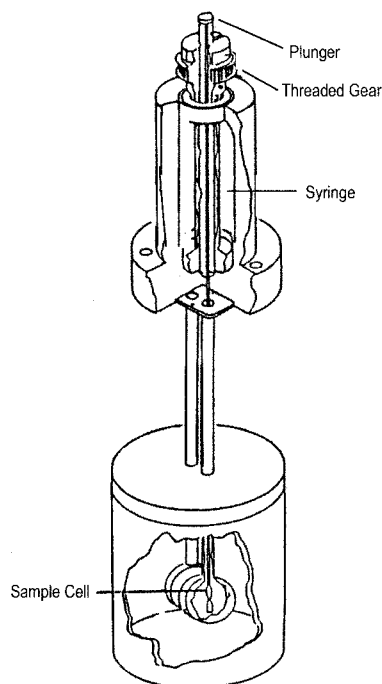


FIG. 1 Diagram of the ITC cell. A modified syringe containing the ligand rotates in place during the ITC experiment. The end of the syringe has been modified to provide continuous mixing. The plunger is computer-controlled and repeatedly injects precise volumes of ligand into the cell containing the macromolecule.

the free-energy change and the enthalpy of binding. For more details see the article by Wiseman *et al.*<sup>9</sup>.

## Use in antibody engineering

Merely measuring the affinity between an antibody and an antigen leaves a great deal of useful and important information unexplored. A full thermodynamic analysis, when possible, provides a richer source of information, which can be used to interpret the molecular forces at work between the antibody and antigen. In one recent example, scientists at Genentech used the instrument described here to carry out thermodynamic investigations of the interaction of a large set of chimaerized anti-HER2 (heregulin) antibody Fab fragments with the p185<sup>HER2-ECD</sup> extracellular domain (ECD)<sup>9-11</sup>. All fragments were single amino-acid variants of the wild-type p185 ECD. The scientists demonstrated that, whereas in some cases affinity constants of the various fragments, determined by RIA or surface plasmon resonance, were essentially identical, their thermodynamic binding parameters ( $\Delta H$  and  $\Delta S$ ) were very different. These differences provided substantial information regarding the specific molecular interactions occurring between the anti-HER2 Fab fragments and the p185 ECD, and formed the basis of further attempts to build stronger and more potent antibodies for use in cancer therapeutics. Perhaps most importantly, these scientists demonstrated that affinity constants alone could not provide enough information to engineer antibody fragments with full wild-type characteristics.

## Characterization and detection

In antibody manufacturing processes, it is critical to identify lot-to-lot variations and variance outside acceptable limits. Typically, antibodies are characterized by measuring their titre alone. Less often, characterization is coupled with the determination of binding constants. It is hoped that titre data provide some indication as to how the antibody product will perform at the end of the manufacturing process. In contrast, ITC provides a full and accurate analysis of the binding interaction. One of the strengths of ITC is that measurements can be done under the same conditions and in the same state in which the antibody will ultimately be used. This is not true in other analytical techniques, where either the antibody or the antigen must be immobilized to a surface before measuring affinity<sup>5</sup>. Another advantage of ITC is its ability to measure affinities in heterogeneous media. It is not necessary to have a fully purified solution in order to detect binding. As long as background affinity is much lower than the affinity one is trying to measure, specific binding against a nonspecific background can be seen.

Heat is accurately described as a 'universal detector' of biological materials and binding phenomena. Unlike optical measurements in which only certain systems may absorb or emit at particular wavelengths, the generation or uptake of heat is a universal signature of all biological processes. Signals obtained by heat detection are comparable in sensitivity to those generated using optical detection. For example, concentrations of biological materials used in ITC experiments are similar to those used in absorbance-based techniques (ultraviolet/visible, analytical ultracentrifugation). In fluorometry, modification of one or both binding partners with a fluorescent tag is typically necessary for detection, sometimes altering the interaction one is trying to investigate. (Note that chemical tagging is physically analogous to surface coupling, which is required for surface plasmon resonance studies.) In RIA, putatively dangerous radioactive compounds are utilized as a signal source. ITC suffers from neither of these limitations.

Modern ITC technology continues to advance. Future instruments will have higher sensitivity and will require less

sample. As has been demonstrated here, ITC is a valuable tool in the physical characterization of antibody products and plays an important role in antibody research laboratories and the antibody manufacturing processes. □

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## ADVERTISEMENTS

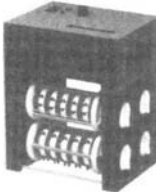
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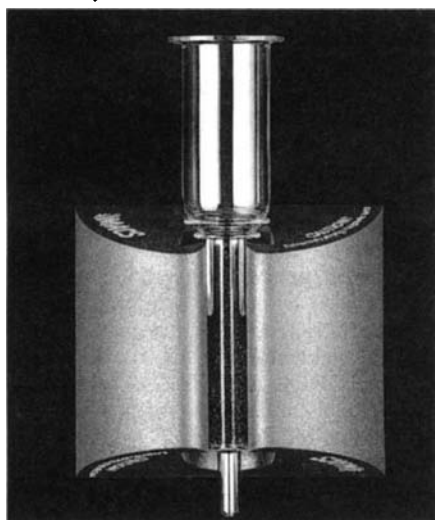
READER ENQUIRY NO. 247



# An open market for cell biology

Cell biologists gather for an annual international meeting in San Francisco this week and items on display may include a non-radioactive assay for cell proliferation, a personal confocal microscopy system or a mammalian expression system.

The new MidiMacs large-scale magnetic cell separation system from Miltenyi Biotec is optimized for the positive selection of larger cell numbers (a stated  $10^8$  cells) from heterogeneous cell mixtures. It is designed for speed, affordability and ease of use with high cell numbers. According to the manufacturer, the extremely small size of the colloidal



Miltenyi Biotec's magnetic cell separator.

MicroBeads (50 nm) makes them well suited for the positive selection of rare cells with frequencies down to  $10^{-8}$ . The beads are biodegradable and do not affect the labelled cell's function or viability. No bead detachment is required. Positively isolated cells can go straight into an experiment and are compatible with flow cytometry, fluorescence microscopy, PCR or FISH analysis. The system is compatible with all of the 50 cell-specific types of Macs MicroBeads.

**Reader Enquiry No. 101**

IBA has screened random peptide libraries systematically in order to design a 'tailor-made' streptavidin binding sequence with improved affinity tag properties, providing one-step protein purification. According to the manufacturer, the resulting nine amino acid Strep-tag (AWRHPQFGG) permits the efficient one-step purification of the protein in question under particularly mild buffer conditions. This purification procedure is said to ensure high yields and can be rapidly carried out, following the standardized operating protocol, which renders special equipment unnecessary. Elution of the bound Strep-tag fusion protein is achieved by simply adding low concentrations of biotin or related com-

pounds, preferably diaminobiotin for repeated use of streptavidin columns, to the physiological washing buffer. According to the manufacturer, these conditions allow the purification of weakly associated protein complexes that carry the Strep-tag at one subunit only. Furthermore, Strep-tagged Fv fragments have been utilized to capture their antigen and to purify the intact Fv-antigen complex. This strategy should prove useful with regard to the rapid isolation of complex membrane proteins. Owing to its small size, the Strep-tag usually does not interfere with the bioactivity of its fusion partner and, therefore, does not need to be removed. In addition, the Strep-tag mediates the convenient detection of recombinant proteins in ELISA, western blot or electron microscopy.

**Reader Enquiry No. 102**

## Cellular scintillation

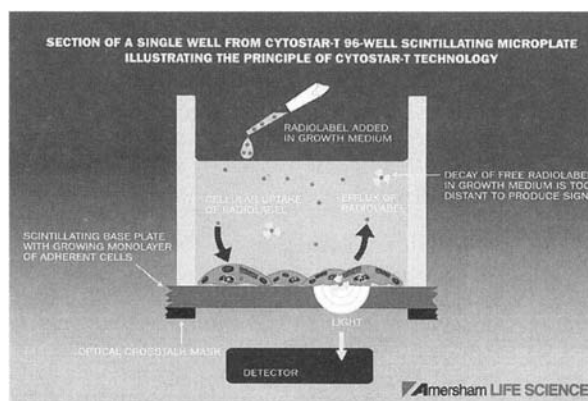
Packard has announced the release of a new brochure covering the TopCount microplate scintillation and luminescence counter — *Expanding the Frontiers of Microsample Analysis*.

The brochure covers the instrument's ability to count up to 12 samples at a time for radioisotopic and luminescent labels, the counting of samples in 384-well microplates, in stacker cassettes (up to 40 microplates), as well as the company's selection of 'crosstalk-free' microplates for a wide variety of applications. The new LucLite and BioLite luminescence assay kits are also introduced in the brochure. These 'glow-type' luminescence assays produce luminescent signals with half-lives in excess of 5 h to overcome the need for built-in injectors. LucLite is used primarily for reported gene assays, while BioLite is used for quantifying cells, particles and microorganisms. The company also offers a topics publication, which describes luminescence assays of living organisms directly in microplates, entitled *Automated Recording of Luciferase-Reported Gene Transcription in Living Seedlings and Fruit Flies*. The study employed repeated, non-destructive luminescence measurements of the transgenic seedlings and fruit flies after exposing them to light for specified

periods of time while on the TopCount instrument. Observation of circadian rhythm as a function of luminescent signal output was observed during several days of measurement.

**Reader Enquiry No. 103**

The Cytostar-T scintillating microplate from Amersham is a non-invasive technology that should find applications in aspects of cell-based drug research, from cell proliferation and receptor-ligand interactions to drug uptake/efflux and cell motility. Developed to improve the scintillation proximity assay (SPA), the plate incorporates scintillant into the base plastic of a standard format 96-well microplate. Thus, cellular events occurring at the cell surface or within cells growing on the base of the wells can be monitored by the addition of relevant radiolabelled molecules to the growth medium. When these molecules bind to, or are taken up by, growing cells, the label is sufficiently close to the scintillant in the baseplate for a signal to be produced. The company has developed methods for studying DNA replication,



Amersham's Cytostar-T scintillating microplate.

receptor-ligand binding, including an assay for G-protein coupled receptors, as well as the flux of  $^{45}\text{Ca}$  through ionotropic channels in neurons. Other applications include the study of the cell cycle, cell growth and apoptosis, and cellular metabolism. However, potential applications for the Cytostar-T microplate include the study of gene expression through mRNA induction. A principal advantage of this plate-based technology is said to be that the cultured cells are not disturbed and, therefore, are analysed under physiological conditions.

**Reader Enquiry No. 104**

## Cell assays

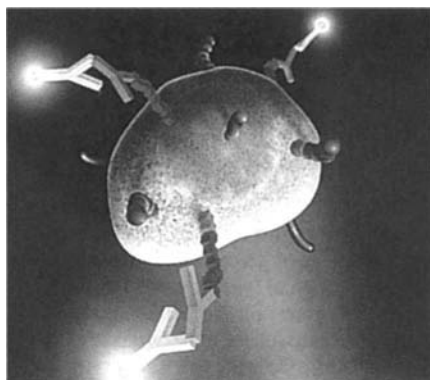
Two safe and convenient, non-radioactive alternatives for the rapid **detection of cell proliferation** have been launched recently by Boehringer Mannheim. The *in situ* cell proliferation kit FLUOS uses fluorescence as a detection method, whereas the *in situ* proliferation kit AP makes use of colorimetric detection. These two kits have been designed for simplicity, using 5-bromo-2-deoxyuridine (BrdU) to identify cells in which DNA synthesis has occurred. BrdU labelling may be carried out *in vitro* and *in vivo*, and is subsequently detected by immunochemical analysis. The elimination of radioisotopes should allow results to be generated more rapidly than with traditional radioisotopic methods. Furthermore, these kits do not require the safety procedures necessary when working with radioisotopes. Reagents in both kits are provided in a stable form, already optimized and quality controlled to give reproducible performance.

**Reader Enquiry No. 105**

Serotec has obtained the rights to market and distribute the Exalpha range of products in the UK, offering a large **range of dual-coloured reagents**. The Exalpha range includes some 90 one-, two- (BiTest) and three-colour (Alpha3) reagents for the research flow cytometry market. The single-colour reagents are conjugated to a number of fluorochromes, including fluorescein (FITC), phycoerythrin (PE), allophycocyanin (APC), AlphaRed or biotin. There is also a range of DNA/RNA reagents for DNA ploidy analysis. These reagents include the NuCycl product line for the extraction and staining of nuclei from cells and paraffin tissue sections.

**Reader Enquiry No. 106**

The Dako Qifikit is intended for the **quantitative determination of cell-surface antigens by flow cytometry** using indirect immunofluorescence assays. The kit consists of a series of six bead populations with well-defined quantities of primary CD5 monoclonal antibodies (mAb). The number of mAb molecules on the six bead populations ranges from zero to 400,000–800,000. The beads mimic cells that have been labelled with a specific mAb. Specimen cells are incubated in parallel with the beads and FITC-conjugated anti-mouse immunoglobulins (F(ab')<sub>2</sub> fragment). A calibration curve is constructed by plotting the fluorescence intensity of the individual bead populations against the number of mAb molecules (antigenic sites) on the beads. The number of antigenic sites on the specimen cells are then determined by interpolation. TallyCal, a Windows-based software program, allows automated conversion of the uncalibrated flow cytometry data into mAb molecules. The beads are run on the



**Qifikit quantifies cell-surface antigens by flow cytometry.**

flow cytometer and both the given mAb values and the recorded MFI at each level are entered into TallyCal. The calibration curve is then used for estimating the antigen density on the specimen cells using the recorded MFI of the cells. This kit has relevance to the quantification of haematopoietic cell antigens involved in haematological malignancies.

**Reader Enquiry No. 107**

## Chemotaxis

Porvair Filtronics has a newly designed **chemotaxis chamber** that accepts 96-well microplates. According to the company, the chamber should create new opportunities for the development of migration studies. Its design allows automated counting of migrating and adhering cells on the bottom of the filter and in the microplate wells. The new design should be useful for studies



**Porvair Filtronics' chemotaxis chamber.**

involving cell types, such as lymphocytes, that typically fall off the bottom of polycarbonate membrane filters after migration. By using standard dimension microplates, the bottom section is compatible with centrifuge buckets, scintillation counters and both ELISA and fluorescence readers. This should simplify post-chemotaxis processing and reading. Fluorescence-stained cells can be read on the bottom of the filter or in the base of the microplate wells, after centrifu-

gation. In the case of radioisotope-tagged cells, these can be collected straight into a white OptiPlate to optimize the sensitivity of the assay.

**Reader Enquiry No. 108**

R&D Systems has recently added **eotaxin** to its existing range of chemokine products. Eotaxin, a potent eosinophil chemo-attractant, is available in both recombinant human and recombinant murine formats. It is a member of the CC chemokine family and specifically binds to the CC chemokine receptor 3 (CCR3). Recent research has suggested a role for eotaxin in HIV infection. The company's recombinant eotaxin is lot-tested for purity and endotoxin levels; its activity is also lot-tested in a human eosinophil chemotactic assay.



**Eotaxin reagent.**

In addition, new anti-chemokine antibodies are also available, including a pan-specific antiGRO monoclonal antibody that can neutralize the activity of human GRO $\alpha$ , GRO $\beta$  and GRO $\gamma$ , as well as monoclonal antibodies against human MCP-2 and MCP-3.

**Reader Enquiry No. 109**

## Optical close-up

The Optiscan **personal confocal system**, available from Axon Instruments in the US, dispenses with the need to mount the confocal elements, including laser and photomultiplier tube (PMT), directly to the microscope. This is achieved through the development of a specialized optical fibre that acts as both the laser delivery and a returning reflection/fluorescence vehicle and, at the same time, provides the confocal pinhole itself. The flexible fibre is made from custom drawn, high-purity silica glass with a 2.5- $\mu$ m diameter germanium-oxide-doped core. Light transmission is stated to be better than 99 per cent using fluorescence wavelengths over the standard 2.5-m length. The fibre interchange, if required, has been simplified by using standard bayonet-type FC connectors. According to the manufacturer, realignment of the system when changing to different microscopes is generally not necessary. The utilization of this fibre to provide the required confocal elements results in a scanning head that is smaller, lighter and more transportable. Other features include an optical-fibre patchcord, which connects the launch/detection unit and a scanning head; the scanning head consists of high-speed galvanometer scanners, fitted with reflective, dielectric-coated silvered mirrors and suitable for patch-clamp applica-

tions (1.7 kg in weight). The launch/detection unit consists of the laser delivery optics, optical-path beam splitters, excitation and barrier filters and photomultiplier tubes. The remote control panel is a small bench-top unit, which controls the photomultipliers, the microscope's focus and the automatic stage movement, where fitted. The system's master control unit contains all control electronics, supplied in an additional PC case. There is also a choice of lasers, a stepper motor, software (Windows 95, Windows for Workgroups or Windows 3.1.x), a configured Intel Pentium PCI local-bus personal computer and a transmission detector, available as an option to allow simultaneous DIC-type image acquisition.

**Reader Enquiry No. 110**

Noran Instruments has released Intervision 1.6 software for its new **OZ confocal laser scanning microscope**. A high-resolution scan mode has been added to support point-scanning and acquisition of  $1,024 \times 960$  images with signal integration. Scan modes for capturing images at video rates and faster (up to one image every two



**Noran Instruments' confocal microscope.**

milliseconds) have been expanded with more available image sizes. The system was designed to meet a broad range of confocal imaging applications in research and imaging facilities. The software includes a suite of tools for multilabel display in two, three and four dimensions. Ratiometric analysis software, for both live (video-rate) and stored images, is a standard feature. The software runs on a Silicon Graphics computer workstation to enable direct digitization of acquired video-rate images, and for interactive manipulation of large stacks of confocal images.

**Reader Enquiry No. 111**

The new modular design of the Leica DM LS **laboratory microscope** is inherited from predecessor models, such as the company's Biomed microscope. It fea-

tures new Delta infinity optics, which offer improved imaging quality, and a heat compensation system, which is designed to prevent partial warming of the microscope. One of the benefits of this microscope is its improved ergonomic design. The curved shape of the Ernest Igl design is one of the features that should provide high stability and improved layout of all controls. Various 'ergomodules' provide further individual adjustment, such as viewing height, so that users can work under fatigue-free conditions.

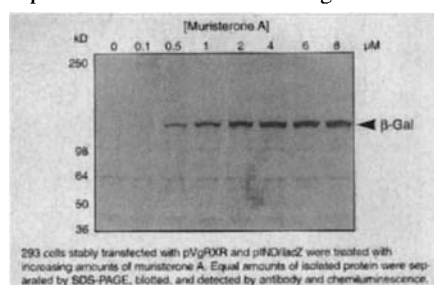
**Reader Enquiry No. 112**

**Mammalian expression**

Clontech is now offering high-level **mammalian expression systems** tightly regulated by tetracycline or its derivative, doxycycline. Tet-Off and Tet-On gene expression provides precisely regulated expression with a stated inducibility of 1,000-fold, allowing both systems to be used for studies involving quantitative regulation of expression, including the expression of toxic genes. Pre-made Tet cell lines are offered by the company and may save weeks of set-up time. Non-academic research laboratories must contact BASF Bioresearch to obtain a licence before purchase.

**Reader Enquiry No. 113**

A new **ecdysone-inducible mammalian expression system** is now offered by Invitrogen. Based on the insect regulatory mechanism, it exhibits almost no basal expression and is stated to have greater than



**Ecdysone-induced expression system.**

200-fold inducibility in mammalian cells. The system also offers dose-dependent control for comprehensive analysis of gene function over a wide range of expression levels. For stable transfection, CHO, 293 and HeLa cell lines that express the regulator protein from pVgRXR are also available. These cells are ready for immediate transfection with the ecdysone-responsive vector, pIND, containing the gene of interest. This system should provide savings in time and effort.

**Reader Enquiry No. 114**

Promega's new pTarget vector systems are designed to provide convenience for researchers **analysing the expression of PCR products in mammalian cells** by

combining the versatility of mammalian expression with the convenience of T-vector cloning and blue/white screening. PCR products generated by enzymes such as *Taq* DNA polymerase typically contain a deoxyadenosine residue on the 3' end. The linear pTarget vector has 3'-complementary T overhangs, allowing direct ligation of PCR products into the vector. Blue/white screening permits the rapid identification of positive clones through disruption of the  $\alpha$ -peptide sequence of the  $\beta$ -galactosidase gene when the gene of interest is successfully inserted. Insert DNA can be readily excised in a single restriction digest using the enzyme *NotI*. Also included is the CMV immediate early enhancer/promoter region, a chimaeric intron downstream of the CMV region and the SV40 late polyadenylation signal. The vector also carries the neomycin phosphotransferase gene, a selectable marker for mammalian cells. The system can be used for transient expression or for stable expression by selecting transfected cells with the antibiotic G-418.

**Reader Enquiry No. 115**

**Culture and media**

Stratagene offers a wide selection of **high-efficiency competent cells**. The cell line includes strains developed to provide cloning solutions for even difficult cloning projects and includes Epicurian Coli XL2-Blue and XL2-Blue MRF'. The manufacturer recommends use of these cells when there are limited amounts of DNA or when using a very large construct. The complete line of competent cells includes speciality strains for a wide variety of applications from cells selected for high efficiency, such as ultracompetent cells, to those selected for reliability, such as subcloning-grade competent cells. The company's competent cells feature a range of cloning efficiencies, intended for use in cloning small amounts of DNA or doing routine day-to-day cloning. Ultracompetent cells provide high-efficiency plasmid library construction, cloning of limited amounts of DNA, transformation of large constructs, or other applications where high transformation efficiency is important. The ultracompetent cells are provided in single-use, 0.1-ml aliquots that are said to eliminate the need for freeze-thaw cycles. Supercompetent cells are available in a wide variety of strains at efficiencies greater than  $1 \times 10^9$  transformants per microgram, and include XL1-Blue, SURE 2, XL1-Blue MRF', XL1-Blue MR and XL1-Blue MRF' Kan. Competent cells are available for cloning procedures that do not require supercompetent efficiencies. At greater than  $1 \times 10^8$  transformants per milligram, this group of competent cells is an economical alternative for routine cloning. Subcloning-grade competent cells are suggested for use when there is no need for high efficiency, but there is still a need for

## PRODUCT REVIEW

consistent day-to-day results. These cells are said to be the economical choice when DNA is not limiting. Electroporation-competent cells should provide ease of use: thaw the cells, add DNA and pulse in the electroporator. Ease of use and high efficiency make electroporation a popular method for library construction, cloning large inserts or when cloning limited amounts of DNA. XL1-Blue, XL1-Blue MRF<sup>+</sup>, SURE and TG1 cells are all available as electroporation-competent cells.

**Reader Enquiry No. 116**

Ex-Cell 610-HSF and Ex-Cell 620-HSF are **serum-free hybridoma media** from JRH Biosciences. Both are low in protein ( $11 \text{ mg l}^{-1}$ ) and support a wide range of cells, including lymphoid, epithelial cells and B-cell hybrids of murine, rat and human origin. Ex-Cell 620-HSF is suitable for DFHR-(HT) selection systems or the GS system. Custom media development, cell-line adaptation and custom manufacturing services are offered by the company to allow researchers to meet their long-term research and manufacturing goals.

**Reader Enquiry No. 117**

According to CellPath, efficiency and **safety in the histology laboratory** are increased with the use of CellStore pots. The pots are pre-filled with buffered formalin or formal saline, ready for immediate use for the safe transportation and storage of samples. The design includes a liquid-tight lid for safety, a wide neck for ease of use and a fully compliant Chip 2 label. There is batch-numbering for easy traceability. Pots are available in quantities of 100 in 60-ml and 90-ml sizes and are packed in convenient, shrink-wrapped trays of 25. Buffered formalin or formal saline can be purchased in bulk, which should prove more cost-effective.

**Reader Enquiry No. 118**

Mediatech and InvitroCytte jointly offer **human primary cells and complete media** for their use. Normal human neonatal fibroblasts are now available as cryopreserved, low passage, primary cells isolated from neonatal foreskin, which are stated to have a Trypan Blue viability upon thawing of greater than 80 per cent. The cells are negative for HIV-1, hepatitis B, bacteria, fungi and *Mycoplasma*. The company provides all the products necessary for cultivating, propagating and subculturing fibroblasts. The complete fibroblast medium system includes fibroblast basal medium and low-serum growth supplement. These products are offered separately in order to give the user flexibility in preparing complete media that is free of antibodies and antimycotics, reducing the chance of microbial contamination going undetected during aseptic filling.

**Reader Enquiry No. 119**

### Reagents

Alexis has released a new research product, **peroxynitrite, for apoptosis research**. This apoptosis reagent may find use in areas of research such as stroke, Alzheimer's disease, Huntington's disease, Lou Gehrig's disease, HIV, encephalopathy, leukaemia and spinal muscular atrophy. Peroxynitrite is a very reactive free radical that has been implicated as the potential trigger of apoptosis, as well as being associated with free radical damage. The company provides 5-ml quantities of peroxynitrite, a free negative control and a complete instruction manual with each order.

**Reader Enquiry No. 120**



**Alexis' peroxynitrite reagent.**

Quantazyme ylg is a high-performance **yeast lytic enzyme** produced by Quantum Biotechnologies, which is a recombinant form of  $\beta$ -1,3 glucanase. The enzyme is purified from an *Escherichia coli* strain carrying the recombinant lytic enzyme from *Anthrobacter* species on an expres-

sion plasmid. The enzyme is purified to be free of protease activity and free of detectable nucleic acids and endo- and exonucleases. According to the manufacturer, it is a highly active enzyme for use in processes requiring digestion of the yeast cell wall, including transformation, protoplast formation, yeast cell disruption, industrial processes and yeast inactivation. It is said to be highly stable, retaining full activity for months in solution at 25 °C. It may be denatured in 8 M urea and will regain full activity upon dialysis. One unit of enzyme is stated to provide lysis of  $0.6 \times 10^6$  yeast cells (*Saccharomyces cerevisiae*) per minute at pH 7.5 and 25 °C. The product is offered in formats ranging from 2,000 U up to 5,000 U.

**Reader Enquiry No. 121**

*These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.*

### Erratum

The review of Axon Instruments' Axopatch 200B patch clamp amplifier, as it appeared in *Nature* (384, 196; 1996), should have read "15 fA (0.1–1.0 kHz) 130 fA r.m.s. (0.1–10 kHz)", instead of "15 fA r.m.s. (0.1–1.0 kHz)".

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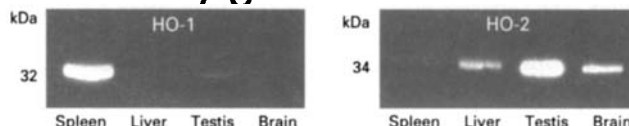
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